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(54) Title: METHOD FOR PREPARING BIOACTIVE POLYMERS

(57) Abstract

A method to prepare a bioactive polymer by covalently binding at least one amino group containing ligand to at least one polymer containing a plurality of free hydroxyl groups, said method comprising the following steps: (i) reacting the at least one polymer with an appropriate activating agent; (ii) reacting the resultant activated polymer in aqueous solution with desired amino group containing ligands; (iii) blocking by reaction or removing by hydrolysis residual polymer bound-ligand unreacted, activating groups; and wherein the said activating agent and/or leaving by-products formed by step (i) and/or by step (ii) and/or by step (iii), are swelling agents of the support polymer.

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# METHOD FOR PREPARING BIOACTIVE POLYMERS

The present invention relates to a method for preparing bioactive polymers by immobilizing amino acid containing ligands on polyhydroxy polymers.

**Protein immobilization methods.**

Intensive efforts have been performed in the scientific community in order to develop new ways to immobilize bioactive materials such as proteins to different polymeric supports. The supports may have different shapes (e.g. beads, nanoparticles, fibers and films) and different chemical and/or physical properties, i.e. rigid, soft, porous and non-porous. The formed optimal bioactive-polymeric conjugates can then be used for extensive applications, i.e. catalysis, specific cell labeling and cell separation, drug delivery, controlled release, cell growth, affinity chromatography, hemoperfusion, etc. (See article by S. Margel *et al* in "Microspheres, Microcapsules & Liposomes", Ed. R. Arshady, Plenum Pub. Company, (1996) in press). Figure 1 describes the major methods commonly used for immobilization of proteins, i.e. enzymes, onto polymeric supports: (A) Attachment of proteins to supports via physical, ionic or covalent forces; (B) Crosslinking of proteins to supports via different crosslinking reagents (i.e. glutaraldehyde); (C) Entrapment of proteins within different support matrixes. These studies are aimed at dealing with the first and second immobilization methods, wherein covalent bonds between the immobilized proteins and the polymers are formed.

**Immobilization methods via covalent forces.**

Usually, the binding between a desired protein and a solid support is based on a chemical reaction between amine groups of the protein and a variety of functional groups of the support. Figures 2-5 describe possible ways to covalently bind amino ligands such as proteins to polymers containing functional groups such as hydroxyls, carboxyls, aldehydes and amines, (see in "Methods in Enzymology", Immobilized Enzymes and Cells, Ed. by K. Mosbach 135, 30-65 (1987)). The present studies however deal with polymers containing hydroxyl groups, particularly cellulose, and

with ways to immobilize proteins to these polymers for applications such as wound healing.

**Immobilization of bioactive compounds with crosslinking reagents.**

A relatively "old" method to immobilize significantly increased amounts of proteins on a polymer support is based on the use of appropriate crosslinking reagents. The immobilization of proteins, i.e. enzymes, is performed by the formation of intermolecular crosslinking between the protein molecules by means of bi- or multifunctional reagents, (see L. Goldstein and G. Manecke, in "Applied Biochemistry and Bioengineering", v. 1, pp. 34-41, Ed. L.B. Wingard, E. Katzir and L. Goldstein, Academic Press 1976). A water-insoluble protein, i.e. enzyme, preparation can be produced by intermolecular crosslinking of the enzyme molecules in the absence, or presence, of a solid support. If the reaction is carried out in the absence of solid supports, this method results in the formation of a three-dimensional network of enzyme molecules. However, in practice enzymes are more usually crosslinked by bi- or multifunctional reagents following adsorption or entrapment within a suitable carrier, i.e. cellulose, silica, polyacrylic acid, polyvinylalcohol, polystyrene, etc., (see in "Immobilized Enzymes" Ed. Ichiro Chibata, Kodansha Ltd. Tokyo, John Wiley & Sons, pp. 46-73 (1978); M.D. Lilly). The common employed crosslinking reagents are glutaraldehyde (Schiff' base), isocyanate derivatives (peptide bond), bisdiazobenzidine (diazo coupling), N,N'-polymethylene bisiodoacetoamide (alkylation) and N,N'-ethylene bismaleimide (peptide bond), (see "Immobilized Enzymes", Ed. I. Chibata, Kodansha Ltd., Tokyo). The functional groups of proteins participating in the reactions include the α-amino group at the amino terminus, the ε-amino group of lysine, the phenolic group of tyrosine, the sulfhydryl group of cysteine and the imidazole group of histidine (see in "Immobilized Enzymes", Ed. I. Chibata, Kodansha Ltd., Tokyo, John Wiley & Sons, pp. 46-49 (1978)).

**Bioactive cellulose.**

Cellulose is a linear polymer of D-glucose residues linked by $\beta(1 \rightarrow 4)$

glycosidic bonds as shown in Figure 6. The hydrogen bonds existing between the cellulose chains give cellulose fibers exceptional strength and make them water insoluble despite their hydrophilicity. The major reactions related to primary and secondary hydroxyl groups can be applied also to cellulose, i.e. oxidation to form dialdehyde cellulose and esterification to form cellulose acetate. Cellulose due to its unique properties, i.e. high hydrophilicity and stability, the presence of hydroxyl groups suitable for protein binding, minimal non-specific interactions, etc., is commonly used for amino ligands (i.e. proteins) immobilization. The binding chemistry of amino ligands to polymers containing hydroxyl groups such as cellulose, polyvinylalcohol, agarose and dextran was intensively investigated and performed according to Figure 2, (see K. Nilsson, K. Mosbach in "Eur. J. Biochem.", 112, 397 (1980), E.V. Groman and M. Wichek in TIBTECH 5, 220 (1987) and J. Kohn and M. Wilchek in "Biochemical and Biophysical Res. Commun.", 107, 878 (1982)). Generally, this reaction is accomplished by adding the dried polymer to an organic solvent (e.g. acetone and dioxane) containing the desired concentration of activating reagent (e.g. tosyl chloride, tresyl chloride, chloroformate, cyanogen bromide, etc.). A base (e.g. pyridine, triethylamine, etc.) is then usually added to the organic solution in order to facilitate the reaction between the activating reagent and the hydroxy-polymer by neutralizing liberated gases such as HCl and HBr. The polymer is then washed with an appropriate organic solvent from an unbound activating reagent. The activated polymer is then interacted in aqueous solution with the desired amino ligand. Unbound ligand is then removed from the bound one. If necessary, residual activated groups are blocked by common ways, e.g. hydrolysis or a reaction with a second amino ligand. The common organic bases used during the activation procedure are pyridine and/or triethylamine. For example, the reaction of primary hydroxyl groups with p-toluene sulfonyl chloride (tosyl chloride) and/or trifluoroethane sulfonyl chloride (tresyl chloride) forms tosylate esters, which have excellent leaving properties in reactions with amino ligands, as shown in Figure 7.

A new method (method 2) for increasing the activity of amino ligands such as proteins immobilized to polyhydroxy polymers such as cellulose was recently described by S. Margel *et al* in U.S.A. Patent 5,516,673. According to this method, bioactive conjugated polyhydroxy polymers with increasing activity were prepared

by a preactivation step in which the polymers are contacted with an N-heterocyclic compound, preferable pyridine, in the absence of activating reagents for hydroxy groups. For example, cellulose may be preactivated by soaking in pyridine as a solvent. The pyridine swollen cellulose is then added to an organic solvent (e.g. acetone or dioxane) containing desired concentration of activating reagent (e.g. tosyl chloride, tresyl chloride, chloroformate, cyanogen bromide, etc.). In this method it is not necessary to add a base (e.g. pyridine or triethylamine) to the activating reagent organic solution due to the presence of excess pyridine in the swollen cellulose. The polymer is then washed with an appropriate organic solvent from an unbound activating reagent. The activated polymer is then interacted in aqueous solution with the desired amino ligand according to the previous method. It was found that in the bioactive amine/polyhydroxy polymer conjugates produced in this manner, the specific activity of the bound amino ligands is in most cases significantly higher than that formed by the known activation method. Figure 8 shows a hypothetical scheme depicting the preactivation of cellulose by pyridine (see in "Cellulose and Cellulose Derivatives (part V)", Ed. N.M. Bikates and L. Segal 719 (1970)). Some other amines such as N-heterocyclic compounds, methylamine and urea are also known to be swelling reagents of cellulose (see N.I. Klenkova in "Zhurnal Prikladnoi Khimii" 40 (10), 2191 (1967); J. C. Thomas in JACS 75,5346 (1953)). The increased activity of amino ligands conjugated to cellulose (or other related polyhydroxypolymers) by the later method (method 2) compared to the known method (method 1) is related to the pre-swelling of these polymers by the pretreatment with pyridine (or other appropriate N-heterocyclic compounds). This swelling phenomena is probably due to the stronger hydrogen bonds formed between the cellulose chains and the preswelling solvent than between each two cellulose chains (OH...N > OH ...O).

A recent patent application (Israel Pat. Appl. 111609, 1994) by S. Margel *et al* described a method (Method 2-I) to significantly increase the binding capacity of amino ligands bound to polyhydroxy polymers such as cellulose by crosslinking the polymer-conjugated protein prepared by methods 1 or 2, particularly by method 2, in the presence of non-conjugated protein by reacting conjugated and non-conjugated protein together in at least one step with crosslinking reagent (i.e. glutaraldehyde), according to the description in Figure 9.

**Wound healing.**

See in U.S. Patent 5,516,673 and Israel Patent Application 111,609 (1994) by S. Margel *et al.*

Wounds may be defined as damage to the skin. A wound may be caused by a scratch on the skin, heat, cold, chemical substances (including radioactive substances), electricity, etc. The term "wound" also includes burns and scars. The skin is one of the most important sensory organs in the body and it is our defensive mechanism against the environment. When part of the skin is damaged, water, salts, proteins and energy are leaked out of the body through the damaged skin. The body loses a significant amount of heat, and bacteria may penetrate into the body through the damaged skin. Fungi and bacteria may cause local contamination in the wound with the threat of deep penetration into the body, resulting in total inflammation. The purpose of treating wounds is to repair the damage caused to the skin. If the damage is small and local, it will usually take a few days or weeks to cure. However, if the damaged area is extensive and severe, the healing process is slow and may require skin implantation and/or other treatments, e.g. drug administration. Often, curing wounds involves severe pain, leaves scars and requires physiotherapy and/or psychological treatment. In severe cases the healing process will have to deal with problems such as bleeding, contamination, pain, poison, water accumulation, etc.

Many methods are currently in use for the treatment of wounds, e.g. antiseptic and antibiotic preparations, laser illumination, cryotechniques, native enzyme preparations, etc. Each method of treatment has both its advantages and disadvantages. Among these methods, the use of native enzymes to treat wounds is well known. Enzymatic preparations are based on enzymes such as proteolytic enzymes (e.g. trypsin, chymotrypsin, etc., which cleanse purulent-necrotic tissues and reduce the amount of pathogens), lysozyme (which dissolves bacteria cell walls), collagenase (which decomposes collagen and prevents formation of rough scars), etc. Enzymatic preparations usually take the form of gels, powders or liquids which are spread on the wound. The use of native enzymes to treat wounds is quite common.

However, this method suffers from some major shortcomings, e.g., native enzymes are rapidly inactivated by inhibitors, they are often (particularly proteolytic enzymes) unstable in aqueous solutions, exhibit antigen and pyrogen properties, may penetrate the blood circulation and thereby cause an allergic reaction and they are also expensive.

In order to overcome these shortcomings, some researchers have covalently coupled various proteolytic enzymes onto polymeric beads of approximately 0.05-0.5 mm average diameter composed of dextran dialdehyde and/or dialdehyde cellulose, (see J. Turkova *et al*, UK Patent Appl. 2147206 (1985)). However, the use of these conjugated beads for treating wounds is limited because of a number of disadvantages, e.g. this method is relatively expensive, quite often the liquid flows from the wounds and sweeps away the conjugated beads, making it necessary to repeat this treatment several times, the beads are biodegradable (see M. Singh *et al* in J. of Biomed. Mat. Res. 15, 655 (1981)) and there is a significant leakage of bound enzymes into the body liquid.

A relatively new method to treat wounds has been developed by Soviet scientists, (see V.V. Rilchev et al HU Patent 207953B (1990); E.O. Medusheva, T.E. Ignatyuk and V.V. Ryltsev in "Khim. Volokna" 3,38 (1992). This method is based on covalent binding of bioactive reagents, e.g. proteolytic enzymes, onto dialdehyde cellulose dressings and/or aldehyde-polycaproamide dressings. Dialdehyde cellulose dressings were formed by periodate oxidation of cellulose and aldehyde-polycaproamide dressings were formed by acidic hydrolysis (3 M HCl) of polycaproamide, followed by glutaraldehyde coupling to the terminal amino groups of the hydrolyzed products. Immobilized enzyme dressings, under the commercial name "PAKS-TRYPSIN" (trypsin bound onto aldehyde-polycaproamide, and DALCEKS-TRYPSIN" (trypsin bound onto dialdehyde cellulose) are commercially available in Russia. These immobilized dressings solved the problem occurring with the previous polyaldehyde beads when quite often the liquid flowing from the wounds swept away the conjugated beads. However, other major shortcomings, i.e. unstable bonds, leakage of bound enzymes into the body liquid, poor mechanical

properties, water solubility, biodegradability and relatively low efficiency for wounds treatment, still exist.

Recently, in order to overcome these shortcomings, novel polymeric surfaces for wounds treatment have been developed in our laboratory, as described by method 2, (see S. Margel *et al* in U.S.A. Patent 5,516,673 (1996)). According to this method, bioactive conjugated polyhydroxy polymers, i.e. cellulose conjugated with trypsin or collagenase, were prepared by a preactivation step in which the polymers are contacted with an N-heterocyclic compound, preferably pyridine, in the absence of activating reagents for hydroxyl groups. The conjugated cellulose surfaces obtained by this activation method (method 2) were significantly more efficient for wound treatment than those obtained by the known activation method (method 1). In order to accelerate the enzymatic action of these new cellulose conjugates, i.e. trypsin coupled to cellulose dressings through method 2, on necrotic wounds a recent patent application (Israel Pat. Appl. 111609, 1994) by S. Margel *et al* described a method (Method 2-I) to significantly increase the binding capacity of amino ligands bound to polyhydroxy polymers such as cellulose by crosslinking the polymer-conjugated protein prepared by methods 1 or 2, particularly by method 2, in the presence of non-conjugated protein by reacting conjugated and non-conjugated protein together in at least one step with crosslinking reagent (i.e. glutaraldehyde), according to the description in figure 9. Thereby, the activity (per weight polymer) and efficiency for wound healing of bioactive dressings prepared by method 2-I is significantly increased relatively to similar bioactive dressings prepared by methods 1 or 2.

Method 2 and thereby method 2-I, however, still suffer from some major shortcomings: (1) The preactivation solvent, particularly pyridine, is toxic and has a disagreeable odor, (see Merck Index, Eleventh Edition, p. 1267). Usually, it is significantly difficult to obtain bioactive polymers free of traces of pyridine, particularly when cellulose is used as a supporting material. Also, scaling up of this system is significantly difficult and the work with pyridine is inconsistent with environmental goals of any workplace; (2) The leaving products formed by the reaction of the amino ligands and the activating polymer are usually toxic (e.g. p-toluenesulfonic acid when tosyl chloride is the activating reagent, (see in Merck

Index, Eleventh Edition, p. 1501) and the release of traces of these products may be hazardous.

The entire disclosures of the literature and patent references mentioned herein, are explicitly incorporated by reference, in the present specification.

The present invention provides in one aspect a method to prepare a bioactive polymer by covalently binding at least one amino group containing ligand to at least one polymer containing a plurality of free hydroxyl groups. The said method including the following sequential steps: (i) reacting the at least one polymer with an appropriate activating agent; (ii) reacting the resultant activated polymer in aqueous solution with desired amino group containing ligands; and (iii) blocking by reaction or removing by hydrolysis residual polymer bound-ligand unreacted, activating groups; and wherein the said activating agent and/or leaving by-products formed by step (i) and\or by step (ii) and/or by step (iii), are swelling agents of the support polyhyroxy polymer.

The amino group containing ligand can be a biologically active protein, while the polyhydroxy polymers can be cellulose, polysaccharides, other polymers containing a plurality of hydroxy groups and their functional derivatives. As examples of activating agents one can include carbonylating agents, such as 1,1-carbonyldiimidazole and 1,1-carbonyldi-1,2,4-triazole.

The products of the invention comprising immobilized protein may for example be fabricated as a powder, bandage, patch or any like cover for application to wounds. However, the invention is not limited in its utility to the treatment of wounds. Thus, the article of the invention may take the form of a powder or column for removing undesired substances from liquid streams, e.g. from the blood by circulating the bloodstream therethrough or by in vitro treatment of serum or other biological fluids. Thus, illustratively, the immobilized protein may comprise e.g. albumin for removal of bilirubin from blood or serum; anti-LDL cholesterol antibodies for removal of LDL cholesterol from blood; or antibodies, antigen or

antiantibody antibodies (as respectively appropriate) for removing a particular antigen or antibodies from blood or other biological fluids.

The biologically active protein may be selected from e.g., trypsin, chymotrypsin, lysozyme, collagenase, albumin and hyaluronidase.

In a particular embodiment of the invention, crosslinking polymer-conjugated protein in presence of non-conjugated protein is effected by reacting conjugated protein with crosslinking agent and non-conjugated protein in successive steps, thereby forming a plurality of crosslinked layers of non-conjugated protein crosslinked also with conjugated protein.

The "functional derivatives" referred to herein include partially etherified and/or esterified derivatives, provided of course that the polymers in question still retain a plurality of hydroxy groups which are capable of reacting in such manner that the bioactive amines can be conjugated to the polymers.

As non-limiting examples of polysaccharides other than cellulose there may be mentioned agarose and dextran. As a non-limiting example of "other polymers containing a plurality of free hydroxy groups" there may be mentioned polyvinyl alcohol and its copolymers e.g. with olefins such as ethylene; modified polyolefins (e.g. polyethylene) such as illustratively grafted polyolefins, containing surface -OH groups.

The crosslinking includes utilizing excess protein in the reaction mixture and/or adding fresh protein thereto. The same protein may be conjugated and crosslinked, or alternatively a mixture of proteins may be conjugated to the polymer and/or crosslinked with conjugated protein(s). Thus, the phrase "crosslinking the polymer-conjugated protein in presence of non-reacted protein" is to be construed broadly in the specification and claims to include all possible alternative modes of operation, including, as mentioned above, forming a plurality of crosslinked layers of non-conjugated protein crosslinked also with conjugated protein. As illustrative crosslinking agents one may select glutaraldehyde and similar materials.

The invention also includes the possibility of conjugating a non-proteinaceous bioactive amine to the polymer, in addition to the protein or proteins. Persons of the art will of course be aware of, for example, pharmacologically active non-proteinaceous amines which could additionally and usefully be conjugated to the polymer. Thus in another aspect the invention provides articles comprising immobilized protein manufactured from at least one polymer containing a plurality of hydroxy groups or their functional derivatives conjugated with at least one amine function of a biologically active protein which is crosslinked with at least one amine function of at least one other biologically active compound.

As an additional optional step prior to the cross-linking step, any residual polymer-bound groups in the polymer-non-crosslinked protein conjugate which are the product of reacting polymer-bound hydroxy groups with the at least one reactant may be reconverted to hydroxy groups, by methods known or available to a person of ordinary skill in the art.

A person of ordinary skill in the art will appreciate that the process including the sequential steps as described herein is a novel process and that the product of such process will constitute a novel product per se, i.e. independently of the process, even where this is not in the form of a cover for application to wounds.

The person of ordinary skill in the art will further appreciate that the present invention makes available a method for the treatment of wounds by applying thereto a powder, bandage, patch or like cover according to the present invention as defined herein. The wounds treated may be burn wounds, but are not limited thereto.

**Materials and Methods.**

**Materials:** Acetone (HPLC) from BIO-LAB Laboratories LTD, Israel; albumin (human, fraction V), gelatin, collagen (acid soluble), trypsin from porcine pancreas-type II, collagenase - type XI, lysozyme from chicken egg white, Na-benzoyl-l-arginine ethyl ester **(BAEE)** hydrochloride, goat anti-human albumin antibodies

serum, lyophilized cells of Micrococcus lysodeikticus from human umbilical cord-sodium salt, Sephadex G-200, p-toluenesulphonyl chloride **(tosyl chloride)**, 2,2,2-trifluoroethanesulfonyl chloride **(tresyl chloride)**, cyanogen bromide, glycine, 4-nitrophenyl chloroformate **(NPC)**, Tris-HCl, agarose, 1,1'-carbonyldiimidazole **(CDI)**, polyoxyethylene with terminal amine functionality (m.w. ca. 3,000) and N-(3-[2-furil]acryloyl)-Leu-Gly-Pro-Ala **(FALGPA)** from Sigma, St. Louis, MO; pyridine, 3-glycidoxypropyltrimethoxysilane, toluene, $BH_3$ in THF (1 M), triethanol amine, triethyl amine **(TEA)**, ethanol amine, Folin-Ciocalteus phenol reagent and glutaraldehyde, 25 wt. % from Aldrich Chemical Company, Inc., USA; $ZnCl_2$, $CoCl_26H_2O$, $CaCl_22H_2O$ NaCl, $NaHCO_3$, NaOH, $KH_2PO_4$, tris(hydroxy-methyl)aminomethane, silica beads of ca. 5 μm, casein for biochemistry and activated molecular sieve 4A from Merck, Darmstadt, Germany; cellulose (cotton) - medical gauze from Central Medical Supply A.R.M., Israel; cellulose powder MN 300 from Duren, Germany; 1,1-carbonyldi-1,2,4-triazole **(CDT)** was formed according to H.A. Staab and K. Wendel, Org. Syn. 48, 44 (1968). Tris-$CaCl_2$ buffer was prepared by mixing 0.05 M Tris-HCl with 0.005 M $CaCl_2.2H_2O$ and 0.2 M NaCl. NaOH aqueous solution (0.1 M) was then added dropwise to the former mixture to reach pH-7.5. HPLC acetone and toluene were dried with molecular sieves 4A and sodium, respectively.

**Preparation of hydroxyl modified silica:** Hydroxy-silica beads were prepared according to P.Wikstrom *et al* in J. of Chromatography, 455,105 (1988). Briefly, 1.8 g silica beads (5 μm average diameter) were dried in vacuum (20 mmHg) for 4 h at 160°C. Dry toluene (300 ml) was added to the silica followed by 1.1 ml of 3-glycidoxypropyltrimethoxysilane and 0.1 ml of the catalyst triethylamine. The slurry was stirred and refluxed under nitrogen for ca. 12 h. The epoxide silica was washed extensively by repeated centrifugations with dry toluene and then acetone. The dried epoxide silica was then converted into diol silica by suspending the silica in water acidified to pH-2.3 with sulfuric acid and stirred at 90°C for 3 h. The hydroxyl modified silica beads were then washed extensively with water and then dried in a vacuum oven.

**Preparation of hydroxyl modified polyethylene:** Hydroxyl modified polyethylene (PE-H) were prepared according to S. Margel *et al.* J. of Polymer Science, Chemistry Edition, 30,1103 (1992). Briefly, ethanol washed PE-H pieces were soaked at 72°C for 5 min in a mixture of chromium trioxide, water and concentrated sulfuric acid in a 3:4:3 weight ratio. The samples were then removed into 70% $HNO_3$ solution at 50°C for 15 min. Then, the oxidized pieces (PE-$CO_2H$) were thoroughly washed with deionized water at 50°C, then with acetone, and dried overnight at room temperature in a vacuum oven. Reduction of the oxidized pieces were performed by soaking these polymer in 1M $BH_3$ in THF solution at room temperature for 12h under nitrogen. The polymer was then rinsed twice in THF, twice in sulfuric acid (4N) at 50°C, twice in water and then with acetone. The reduced pieces thus obtained (PE-$CH_2OH$) were dried at room temperature in a vacuum oven.

**Experimental animals:** Female guinea-pigs from Anilab., Rehovot, Israel, weighing 300-350 g were conditioned for two weeks prior to experiments. The animals received water and basal diet without antibiotics and were housed in controlled temperature (23 - 25°C) and light and dark (12 h/12 h LD).

**Methods of analysis**:

**A.** Swelling degree of cellulose by different reagents was determined by measuring the change in volume of cellulose powder as a consequence of contact between the cellulose and these reagents. For this purpose, 2 ml of an appropriate solvent (i.e. acetone or pyridine) or 2 ml of acetone solution containing the studied reagent (i.e. CDI in acetone) were added to an NMR tube (5 mm diameter) containing 0.1 g of cellulose powder. The formed suspension was shaken intensively and then left to stand vertically for 24 h. The change in the volume of the cellulose in the tubes was then measured.

**B.** Thermogravimetric analysis (TG) was performed with the equipment of Mettler, Toledo, Swizerland.

**C.** Hygroscopicity degree of the activated cellulose was measured by placing a drop of water (10 µl) on the surface of a dressing or a pellet prepared from activated cellulose powder and immediately observing the angle formed between the drop and the cellulose surface, (see S. Brandriss and S. Margel, Langmuir 9, 1232 (1993)).

**D.** Amounts of polymer-bound imidazolyl or tosyl groups were derived from the absorption peaks at 224 nm and 261 nm, respectively, obtained after hydrolysis of 50 mg activated polymer in 5 ml $NaHCO_3$ aqueous solution (0.05 M at pH - 11.0) at room temperature for 1.5 h.

**E.** The amount of protein (enzyme) coupled to the polymers was measured by the method of Lowry *et al.*, J.Biol.Chem., 193, 265 (1951).

**F.** Enzyme activity of coupled trypsin was measured by using several substrates: BAEE, casein and gelatin gel.

**F-1.** Conjugated trypsin activity towards BAEE was measured according to K.A. Walsh and P.E. Wilcox, in "Methods in Enzymology" 19, 31-41 (1970). Briefly, 25 mg of conjugated cellulose is placed in 3 ml 0.1 M phosphate buffer (pH-7.5) containing 0.74 mg BAEE. The mixture is then incubated at 25° C for 25 min. The substrate solution is then analyzed at 253 nm. One BAEE unit activity produces an increase in $A_{253}$ of 0.001 per min per g cellulose at pH 7.5 at 25°C, reaction volume 3.0 ml.

**F-2.** Conjugated trypsin activity towards casein was measured according to M.L. Anson, J. Gen. Phys. 22, 79-89 (1938). Briefly, 25 mg of conjugated cellulose is placed into 0.1 M $NaHCO_3$ aqueous solution (pH-8.5) containing 1% (w/v) casein. The mixture is then incubated at 37° C for 1 h. Thereafter, 1 ml of 7.2% trichloroacetic acid aqueous solution is added and the mixture is incubated for another 30 min. The substrate mixture is then centrifuged at 10,000 rpm for 2 min. The remaining casein fragments in the supernatant are then analyzed at 280 nm.

**F-3.** Conjugated trypsin activity towards gelatin gel.

Gelatin suspension (5% w/v) in phosphate buffer 0.1 M pH 7.5 was heated at 60°C for 20 min. The formed clear gelatin solution was poured into plastic dishes (22 mm diameter, 2 ml/dish) and then allowed to gel at room temperature. The weight of the gel in each dish was then accurately determined.

The dressings were cut into discs of approximately 1.5 cm diameter. Two discs (two layers of dressings) were used on each dish containing the gelatin gel. The disc dressings (50 mg) were applied on the gel surface and then 1 ml of 0.1 M phosphate buffer (pH-7.5) was added to the dressings in each dish. In order to demonstrate the stability of the gelatin gels towards the experimental conditions, 1 ml of phosphate buffer was also added on the gel surface and incubated during the experiment period of time with the test dishes (negative controls).

Samples were incubated for ca. 22 h at 25°C. Following incubation, the solubilized product was poured from the residual gelatin gel into a labeled vessel. The remaining gelatin gel was then washed twice with 1 ml phosphate buffer and the supernatant was also added to the labeled vessel. The solubilized liquid was then assayed for gelatin peptides and the weight of residual gelatin gel was measured. The enzymatic degradation of gelatin caused by trypsin coupled to the dressings was then calculated.

**G.** Enzyme activity of coupled collagenase was measured by using several substrates: FALGPA and gelatin gel.

**G-1.** Conjugated collagenase activity toward FALGPA.

50 mg conjugated cellulose is added into 3 ml of Tris-$CaCl_2$ buffer (pH-7.5) containing 0.35 mg FALGPA. The mixture is then incubated for 1 h at room temperature. The decrease in absorbance at 342 nm (due to the cleavage of the substrate) is then measured. One unit activity produces a decrease of 0.001 per h per g cellulose.

**G-2.** Conjugated collagenase activity towards gelatin gel.

Conjugated collagenase activity towards gelatin gel is measured in the same way as described for conjugated trypsin except that instead of phosphate buffer Tris-$CaCl_2$ buffer (pH-7.5) was used.

**H.** Enzyme activity of coupled lysozyme was measured according to D.F. Ollis and R. Datta, in "Methods in Enzymology" 44, 444-450 (1976) using dried cells of Micrococcus lysodeikticus as substrate. One unit activity of lysozyme is considered as the decrease in $A_{450}$ of 0.001 per min per g cellulose as a result of lysis of the corresponding substrate under experimental conditions (pH 6.24, 25°C, 25 mg cellulose, 0.3 mg/ml suspension of cells, reaction volume 3 ml).

**I.**. The activity of coupled albumin was measured according to L. Marcus et al, J. of Biomed. Mat. Res. 18, 1153 (1984) by removal of goat anti-human albumin from immunized serum via a column containing human albumin bound to cellulose.

**Cellulose washing:**

1 g cellulose (cotton gauze and/or powder) was boiled for 30 min in 25 ml of 1% $NaHCO_3$ aqueous solution . The mixture was then cooled to room temperature, the cellulose then washed extensively with distilled water, dried at room temperature and stored until used.

**Preparation of activated cellulose:**

A. **Method 1** - activation without the preactivation step (known activation method):

Cellulose activation was performed according to the literature, (see E.V. Groman and M. Wilchek, TIBTECH 5, 220 (1987); S. Margel and S. Sturchak, U.S. Patent No. 5,516,673 (1996)) .

Activation with tosyl chloride:

In a typical experiment, 1 g cellulose (not-treated or treated by boiled $NaHCO_3$ as previously described) was washed with 10 ml of water. The water was then removed by drying at 105°C or by washing with water/acetone (3:1, v/v), water/acetone (1:3, v/v), acetone and finally several times with dried acetone. The cellulose was then transferred to a flask containing appropriate amount of tosyl chloride (i.e. 1 g) dissolved in 10 ml dried acetone. During shaking, 2 ml pyridine was added dropwise for about 1-2 min. After a 1 h reaction at room temperature the cellulose was washed extensively with dried acetone and then air-dried or transferred back to water by reversing the washing scheme described above.

Activation under different conditions:

The previous procedure was repeated under different conditions, for example, changing the activation reaction time or concentration of the activating reagent, or substituting the hydroxyl activating reagent (tosyl chloride) with other reagents such as tresyl chloride, cyanogen bromide and p-nitrophenolchloroformate (PNC), or substituting the solvent, i.e. acetone with dioxane, or substituting the base, i.e. pyridine with triethyl amine, or by carrying out the activation without addition of any base.

B. **Method 2**- activation including preactivation step by swelling reagents of U.S. Patent 5,516,673.

Cellulose activation was performed according to S. Margel and S. Sturchak, U.S. Patent No. 5,516,673 (1996).

Activation with tosyl chloride:

In a typical experiment, 1 g dried cellulose was soaked in 2 ml pyridine at room temperature for approximately 30 min. The pretreated pyridine swollen cellulose was then transferred to a flask containing 1 g tosyl chloride dissolved in 10 ml dried acetone. After approximately 1 h reaction at room temperature, the cellulose was washed extensively with dried acetone and then air-dried or transferred to an aqueous solution as described previously.

Activation under different conditions:

The previous procedure was repeated under different conditions, for example, substituting the hydroxyl activating reagent (tosyl chloride) with tresyl chloride, or substituting the solvent, i.e. acetone with dioxane, or substituting the intercalating reagent, i.e. pyridine with pyridazine.

**Method 3-** activation by CDI (activation in presence of swelling activating reagent and/or leaving product).

In a typical experiment, 1g cellulose was shaken for 30 min at room temperature with 10 ml acetone solution containing 100 mg CDI. The activated cellulose was then washed extensively with dried acetone and then air dried or transferred back to water by reversing the washing scheme described above.

The previous procedure was repeated under different conditions, for example, changing the activation reaction time or concentration of the activating reagent, or substituting the CDI with other similar cabonylating reagents such as 1,1-carbonyldi-1,2,4-triazole (CDT), or substituting the cellulose with other polyhydroxy polymers such as hydroxyl modified silica beads, Sephadex and polyvinyl alcohol, or substituting the solvent, i.e. acetone with dioxane, or adding a base such as pyridine or triethyl amine to the acetone solution.

**Binding amino ligands to the activated polymers:**

In a typical experiment, dried activated polymer (e.g. 1 g) was added to an aqueous solution (e.g. 10 ml of 0.1 M bicarbonate buffer at pH 8.5 in the presence or absence of salts such as $CaCl_2$, $ZnCl_2$ and $CoCl_2$) containing the desired amino ligand (e.g. 10 mg trypsin). The binding reaction was performed, usually, at room temperature for the desired period of time. The conjugated polymer was then washed in aqueous solution from unbound amino ligand. If necessary blocking of residual bound activating reagent was then performed by hydrolysis or by binding a second amino ligand (e.g. ethanol amine) to the conjugated polymer. Unbound ligand was then removed by extensive washing with bicarbonate buffer and then with saline. The bioactive polymer was then washed fast with distilled water and then air-dried.

**Binding amino ligands to the conjugated cellulose by crosslinking reagents:**

Amino ligands were bound to conjugated polymers prepared by methods 1, 2 or 3 according to Israel Patent Appl.111609 (1994) by S. Margel *et al.* In a typical example, 1 g trypsin conjugated cellulose dressing was soaked at room temperature for 5 min in 2.5 ml of 0.1 M aqueous bicarbonate buffer pH 8.5 containing 4 % (w/v) trypsin. After 5 min, 2.5 ml of 0/066 M aqueous buffer solution pH- 6.2 containing 1 % glutaraldehyde was added and the incubation was continued at room temperature for 18 h. Unbound trypsin was then removed by extensive washing with bicarbonate buffer and then with saline. The trypsin cellulose dressing was then quickly washed with distilled water and then air-dried.

**Burn wounds:**

The skin on the back and right side of each guinea pig was prepared for wounding by removing the hair by shaving and with a depilatory cream and then washing the skin with water. Soap and antiseptics were not used because of their potential influence on the wound healing process. Burn wounds were made according to the methodology of S.C. Davis, P.P. Mertz and W.H. Eaglstein, J. Surg. Res., 48, 245 (1990).

On the day of burning, the guinea pigs were anesthetized with ether according to the methodology of J.S. Paterson in " The UFAW Handbook on Care and Management of Laboratory Animals", Ed. by UFAW, 223-241 (1972).

Animals were placed in a ventilated glass container with a pad soaked in ether. Care was taken that the pad did not touch the animal, as ether burns mucous membranes. The animals can thus be observed and be removed when the desired stage is reached.

A specially designed brass rod weighing 45 g was heated to precisely 150°C. The brass rod was held perpendicularly on the right side of the guinea pig's back,

with all pressure supplied by gravity for 10 seconds to make a burn wound of 10 x 20 mm and 0.7 mm deep.

**Figure ligands.**

Fig. 1. A scheme describing different immobilization methods of proteins to polymers.

Fig. 2. A scheme describing possible ways to bind amino ligands to polymers containing hydroxyl groups.

Fig.3. A scheme describing possible ways to bind amino ligands to polymers containing carboxylic groups.

Fig. 4. A scheme describing possible ways to bind amino ligands to polymers containing aldehyde groups.

Fig. 5. A scheme describing possible ways to bind amino ligands to polymers containing amine groups.

Fig. 6. A scheme describing the primary structure of cellulose.

Fig. 7. A scheme describing the reaction of a polymer with hydroxyl groups with amino ligands via activation with tosyl chyloride and tresyl chloride.

Fig. 8. A hypothetical scheme describing the inclusion of pyridine in cellulose.

Fig. 9. A scheme describing crosslinking of proteins to protein-conjugated polymers by one step and two steps coupling reactions.

Fig. 10. A scheme describing the activation of polymers containing hydroxyl groups by CDI.

Fig. 11. A scheme showing the major steps for binding amino ligands to cellulose by the different methods.

Fig. 12. Thermogravimetric analysis diagrams of cellulose and activated cellulose prepared by the different methods.

Fig. 13. Effect of lysozyme concentration on the activity of lysozyme bound to cellulose prepared via methods 2 and 3 at pH 7.5 and 6.3.

**Detailed description of the invention.**

The present invention describes a method to obtain bioactive conjugated polyhydroxy-polymers with high biological activity and high efficiency for wound healing applications. The polyhydroxy-polymers may be chosen from polymers such as cellulose, cellulose derivatives, dextran, polyvinyl alcohol, etc. The bioactive substances are ligands containing amine functionality such as α-amino acids, proteins, enzymes, etc. The preparation of these materials includes the following sequential steps: (1) Reacting a suitable polyhydroxy-polymer with an organic solution containing an appropriate activating reagent such as the carbonylating reagents CDI and/or CDT; (2) Reacting the resultant activated polymer in aqueous solution with desired amino ligands; (3) Blocking residual bound activated groups. For residual carbonylated groups, this may be performed by hydrolyzing these groups under basic aqueous solution to form the original hydroxyl groups, or by reacting these groups in an aqueous solution with other amino ligands, i.e. ethanol amine, glycine, polyoxyethylene with terminal amine functionality, etc. These blocking reaction step may effect the nature of the polymeric matrix, e.g. increase its hydrophylicity by blocking residual bound activated groups with polyoxyethylene.

The major demand of this invention is that the activating reagent and/or the leaving products (formed by the reaction of the activating reagent and the hydroxyl groups of the polymer and/or by the reaction of the amino ligand with the bound activated groups on the polymer and/or by the hydrolysis of residual bound activated groups) are swelling reagents of the support polymer. For example, CDI is a suitable

activating reagent for polyhydroxy polymers such as cellulose but not for hydroxyl modified silica or polyethylene (prepared according to the description in Materials and Methods), since cellulose, but not hydroxyl-silica, swells by contact with organic solution of CDI and/or imidazole (the leaving product). [CDI is an imidazole derivative of urea. It is well established that both components, urea and imidazole (N-heterocyclic compound), are swelling reagents of cellulose, see N.I. Klenkova in "Zhurnal Prikladnoi Khimii" 40 (10), 2191 (1967); J. C. Thomas in JACS 75,5346 (1953) and S. Margel et al U.S. patent 5,516,673 (1996). It is therefore reasonable to postulate that CDI is also a swelling reagent of cellulose].

A further invention of this patent application includes the contact of the previously formed bioactive conjugated matrix containing at least one amine function with non-conjugated protein in at least one step with crosslinking reagent such as glutaraldehyde. The former reaction is performed in aqueous solution for a sufficient period of time to bind covalently the protein to the former bioactive conjugate. The formed bioactive polymers possess increased biological activity and efficiency for treatment of wounds.

The bioactive polymer may for example be selected from a powder, bandage, patch or like cover for application to wounds. However, the invention is not limited in its utility to wound healing. Thus, the article of the invention may take the form of a powder or column for removing undesired substances from liquid stream, e.g. from the blood, by circulating through the blood stream or by in vitro treatment of serum or other biological fluids. Illustratively, the immobilized protein may comprise, for example, albumin for removal of bilirubin from blood or serum; chelating agent for removal of heavy metal ions from blood, serum or aqueous solutions.

The biologically active protein may be selected from, for example, trypsin, lysozyme, collagenase and albumin. The activating reagents may be selected from carbonylating reagents such as CDI and/or CDT. The polyhydroxy polymers may be selected from cellulose, dextran and polyvinyl alcohol.

Method 1 describes the activation of polyhydroxy polymers through its hydroxyl groups by known methods, followed by reaction of amino ligands such as proteins and α-amino acids with thus-formed derivatives to obtain amino ligand/polymer conjugates. This method has been previously described as *a priori* art in U.S. Patent 5,516,673 by S. Margel *et al.* The conjugated polymers, i.e. cellulose covalently coupled with lysozyme and/or collagenase possess relatively low activity and are not efficient for wound healing applications as described in U.S. Patent 5,516,673.

Method 2 is similar to method 1, described above, except that an additional preactivation step using N-heterocyclic compound is carried out, as set forth in U.S. Patent 5,516,673 by S. Margel *et al.* The N-heterocyclic preactivated reagents swell polyhydroxy-polymers such as cellulose by inclusion in between the cellulose chains as shown in Figure 8. Thereby, the distance between adjacent chains and the liquid character of the polymers increased, resulting in increased biological activity of the protein conjugated polymers formed by this method. U.S. Patent 5,516,673 also demonstrates that pyridine is the most suitable N-heterocyclic swelling reagent as shown by the increased biological activity and suitability for wound healing of bioactive cellulose conjugates formed according to this method by pretreatment with pyridine.

Method 2-I describes a new way to obtain bioactive polyhydroxy polymers with increased efficiency to treat wounds as set forth in Israel Patent Application No. 111187 (1994) by S. Margel *et al.* These bioactive polymers were formed by reacting a crosslinker, e.g. glutaraldehyde, with amine groups of protein conjugated to polyhydroxy polymers prepared by methods 1 or 2, particularly by method 2. The formed materials possess significantly increased amounts of bound bioactive reagent (i.e. trypsin) covalently coupled to the polyhydroxy polymer; and increased efficiency for treatment of wounds.

Method 2 and thereby method 2-I, however, still suffer from some major shortcomings: (1) The preactivating swelling solvent, particularly pyridine, is toxic and has a disagreeable odor, (see Merck Index, Eleventh Edition, p. 1267). Usually,

it is significantly difficult to obtain bioactive polymers free of traces of pyridine, particularly when cellulose is used as the support material. Also, scaling up of this system is significantly difficult and the work with pyridine is inconsistent with environmental goals of any workplace; (2) The leaving products formed by the reaction of the amino ligands and the activated polymer are usually toxic (e.g. p-toluenesulfonic acid when tosyl chloride is the activating reagent, (see Merck Index, Eleventh Edition, p. 1501) and release of traces of these products may be potentially hazardous.

This invention aims at preparing efficient bioactive polymers for wound healing application which do not suffer from the former shortcomings. Figure 10 illustrates the reaction of polyhydroxy polymers with CDI to obtain bioactive conjugates. This reaction was intensively investigated by M.T.W. Hearn, (see for example M.T.W. Hearn in J. of Chromatography, 185, 463 (1979); M.T.W. Hearn and E.L. Harris in J. of Chromatography 218, 509 (1981); M.T.W. Hearn in J. of Chromatography 376, 245 (1986)). These studies indicated that two possible activated products may be formed by this reaction: imidazoylcarbonate and cyclic carbonate (derivatization via two proximal hydroxyl groups). The cyclic carbonate may be formed between proximal hydroxyl groups of the same polymeric chain or between adjacent chains. The latter case will result in crosslinking of the polymer. Indeed, under conditions of high activation level a shrinkage level of 15%-20% of some soft gels were noticed by Hearn *et al* in " Methods and Enzymology" 135, 102 1987. Bioactive conjugated polymers suitable for affinity chromatography purposes may be formed by the reaction of these activated polymers with amino ligands (see Figures 2 and 10). Blocking of residual bound activating reagent may be performed by hydrolysis or by a reaction with a second amino ligand as shown in Figure 10. The carbamate bonds (urethane linkage) formed by the reaction of the amino ligands and the hydroxyl groups of the polymer are stable under physiological conditions and therefore leakage of the bound amino ligand into the aqueous working solution is usually not detected. Other important advantages of the activation with CDI compared with other standard activating reagents, e.g. cyanogen bromide, tosyl chloride, tresyl chloride and p-nitrophenyl chloroformate, are the absence of hydrophobic groups and/or charged groups in the activated product, the ease of

handling of CDI, the stability of the activated product and the relative non-toxicity of the product (imidazole) released by the reaction of the polyhydroxy polymer with the activating reagent and by the reaction of the activated polymer with the amino ligand.

This invention, however, related only to some special cases wherein the activating reagent and/or the leaving products (formed by reaction of the activating reagent and the hydroxyl groups of the polymer and/or by reaction of the amino ligand with the polymer bound activating groups and/or by the hydrolysis of residual bound activated groups) are swelling reagents of the support polymer. For example, CDI is a suitable activating reagent for polyhydroxy polymers such as cellulose but not for hydroxyl modified silica or polyethylene (prepared according to the description in Materials and Methods), since cellulose, but not hydroxyl-silica or hydroxyl-polyethylene, swell from contact with an organic solution of CDI and/or imidazole (the leaving product), resulting in substantial expansion (but not shrinkage) of the formed activated cellulose. Bioactive polyhydroxy polymers such as cellulose prepared via this method (method 3) have also significantly increased activity and efficiency for wound healing purposes.

Figure 11 is a scheme showing the major differences in forming bioactive conjugates from polyhydroxy polymers such as cellulose via the different activation methods. Bioactive polymers prepared by method 1 are usually not efficient for wound healing applications and have lower specific activity than bioactive polymers prepared by methods 2 or 3. Bioactive polymers such as cellulose prepared by method 2 required a preactivation step in which the polymer is soaked in an N-heterocyclic compound, preferable pyridine, in the absence of an activating reagent for hydroxyl groups. This extra problematic pretreatment swelling step does not exist when preparing bioactive cellulose via method 3. In method 3 the activating reagent, e.g. CDI, and/or the leaving products, e.g. imidazole, formed by the reaction of the activating reagent with the polymer hydroxyl groups and by the reaction of the amino ligand with the polymer bound activated groups are swelling reagents of cellulose. As a consequence of this effect the swelling of cellulose is only local, i.e. in the

reaction location sites, contrary to the swelling of cellulose by pyridine (methods 3 requires at least 10 times less swelling reagent than method 2). This local swelling effect and the possible crosslinking, due to cyclic carbonate formation between proximal chains, will probably result in activated polymers and bioactive polymers prepared by methods 2 and 3 of different structures and properties. For example, the TG diagram of activated cellulose prepared by method 2 appears at approximately 100° C lower than that prepared by method 3 (see fig. 12 and example 12). These differences in structure and properties result also in different activity and efficiency of bioactive cellulose conjugates prepared by methods 2 and 3. Usually bioactive cellulose prepared via method 3 have a similar or higher activity and efficiency for wound healing applications than that prepared by method 2.

Another major difference between the different methods is that in methods 1 and 2 the presence of a base, i.e. pyridine or triethylamine, during the activation step is usually essential in order to facilitate the activation reaction by removing released acids. On the other hand, the presence of a base while preparing the activated polymer via method 3 is commonly not desirable since it usually reduced the activity and efficiency for wound healing applications of the formed bioactive polymers. This effect is probably due to partial hydrolysis of the carbonylating activating reagents, i.e. CDI, due to the presence of a base.

**Examples.**

**Example 1. Immobilization of proteins to cellulose via CDI (method 3) and via other different activating reagents (method 1).**

A. Immobilization with tosyl chloride.

0.25 g of dry cellulose (medical gauze) pre-washed with with $NaHCO_3$ was treated at room temperature for 1 h with 5.0 ml acetone solution containing 0.5 mmol tosyl chloride. In the experiments where a base was used during the activation time, 1 ml of triethylamine was added dropwise during the first 1-2 min of the the activation period into the acetone solution. The cellulose was then washed extensively with acetone and then air dried. The dried cellulose was then treated

with 2.5 ml bicarbonate buffer (0.1 M, pH-8.5) containing 5 mg trypsin (or 12.5 mg lysozyme) for 24 h at room temperature. The cellulose was then washed from unbound protein by extensive washing with bicarbonate buffer, saline and then air-dried.

B. Immobilizattion with tresyl chloride.

The immobilization of proteins to cellulose via tresyl chloride activation was performed through a procedure similar to the prodedure described in (A) substituting the tosyl chloride for tresyl chloride (0.5 mmol).

C. Immobilization with 4-nitrophenyl chloroformate (NPC).

The immobilization of proteins to cellulose via NPC activation was performed through a procedure similar to the prodedure described in (A), substituting the tosyl chloride for NPC (0.5 mmol).

D. Immobilization with CNBr.

The immobilization of proteins to cellulose via CNBr activation was performed through a procedure similar to the prodedure described in (A), substituting the tosyl chloride for CNBr (0.2 mmol).

E. Immobilization with CDI.

The immobilization of proteins to cellulose via CDI activation was performed through a procedure similar to the prodedure described in (A), substituting the tosyl chloride for CDI (0.31 mmol).

Tables 1 and 2 demonstrate that the activity of the protein conjugated cellulose dressings (per gram cellulose or per mg bound enzyme) prepared in presence of CDI is usually significantly higher than that prepared in the presence of other common activating reagents. This activity ratio between the different enzyme conjugated cellulose dressings prepared via different activating reagents were repeated under different experimental conditions, e.g. changing the concentration of the activating reagents and/or enzymes, changing reaction time, etc. Tables 1 and 2 represent, however, the conditions wherein optimal specific activity of the conjugated dressings

were obtained. It should be noted that increasing the activating reagent concentration may sometimes increases the amount of bound enzymes per each gram polymer. However, the increased amount of bound enzyme per each gram of polymer, due to steric effects, does not necessrily leads to increase in specific activity of the bound enzymes.

**Example 2.**

Example 1 was repeated substituting the cellulose dressings for cellulose powder. Similar differences in the activity ratio of the bioactive cellulose prepared with CDI and the bioactive dressings prepared via the other activating reagents were obtained.

**Example 3.**

Example 1 was repeated substituting the solvent acetone for dioxane. Similar differences in the activity ratio of the bioactive cellulose prepared with CDI and the bioactive dressings prepared via the other activating reagents were abtained.

**Example 4.**

Examples 1 - 3 were repeated substituting cellulose that was pre-washed with 1% $NaHCO_3$ for cellulose that was pre-washed with $H_2O$. Similar differences in the activity ratio of the bioactive cellulose prepared with CDI and the bioactive dressings prepared via the other activating reagents were abtained.

**Example 5.**

Examples 1 - 4 were repeated substituting trypsin and lysozyme for albumin and omitting the experiments with NFC and CNBr. Similar differences in the activity ratio of the bioactive cellulose prepared with CDI and the bioactive dressings prepared via the other activating reagents were abtained.

**Example 6.**

Examples 1 - 4 were repeated substituting CDI for CDT. Similar differences in the activity ratio of the bioactive cellulose prepared with CDT and the bioactive dressings prepared via the other activating reagents were abtained.

**Example 7.**

Examples 1 - 3 were repeated substituting cellulose for Sephadex G-200. Similar differences in the activity ratio of the bioactive cellulose prepared with CDI and the bioactive dressings prepared via the other activating reagents were abtained.

**Example 8.**

Examples 1 - 3 were repeated substituting cellulose for hydroxyl-silica beads and omitting the experiments with NFC and CNBr.
The specific activity of the bioactive hydroxy-silica beads prepared via CDI was similar to that prepared via tosyl chloride and tresyl chloride.

**Example 9.**

Examples 1 - 3 were repeated substituting cellulose for hydroxyl-polyethylene and omitting the experiments with NFC and CNBr.
The specific activity of the bioactive hydroxy-polyethylene prepared via CDI was similar to that prepared via tosyl chloride and tresyl chloride.

**Example 10. Swelling level of cellulose in different environments.**

The swelling level of cellulose by different reagents was determined by measuring the change in volume of cellulose powder as a consequence of contact between the cellulose and these reagents. For this purpose, 2 ml of an appropriate solvent (i.e. acetone or pyridine) or 2 ml of acetone solution containing the studied reagent (i.e. CDI in acetone) were added to an NMR tube (5 mm diameter)

containing 0.1 g of cellulose powder. The formed suspension was shaken intensively and then left to stand vertically for 24 h. The change in the volume of the cellulose in the tubes was then measured.

Table 3 demonstrates that cellulose does not swell (significantly) by acetone. On the other hand, a significant swelling of cellulose by CDI and by the N-heterocyclic compounds, pyridine and imidazole, is observed.

**Example 11. Swelling level of hydroxy-silica and hydroxy-polyethylene in different environments.**

Example 10 was repeated substituting cellulose for hydroxy-silica or hydroxy-polyethylene. A significant swelling of the hydroxy-substrates by the different reagents (i.e. acetone, pyridine, $H_2O$, CDI and imidazole) was not observed.

**Example 12. Thermogravimetric analysis diagrams of cellulose and activated cellulose prepared by methods 1 - 3.**

Cellulose dressings were activated via methods 1 - 3 , according to the following procedures:

Method 1

1 g dried cellulose was transferred to a flask containing 1 g tosyl chloride dissolved in 10 ml dried acetone. During shaking, 2 ml pyridine was added dropwise for about 1-2 min. After a 1 h reaction at room temperature the activated cellulose was washed extensively with dried acetone and then air-dried.

Method 2

1 g dried cellulose was soaked in 2 ml pyridine at room temperature for approximately 30 min. The pretreated pyridine swollen cellulose was then transferred to a flask containing 1 g tosyl chloride dissolved in 10 ml dried acetone. After approximately 1 h reaction at room temperature, the activated cellulose was washed extensively with dried acetone and then air-dried.

Method 3

1g dried cellulose was transferred to a flask containing 100 mg CDI in 10 ml of dried acetone. After 1 h reaction at room temperature the activated cellulose was washed extensively with dried acetone and then air-dried.

Table 4 and figure 12 demonstrates the different behavior of activated cellulose prepared by methods 1 - 3. The Dpeak (point of bend) temperatures of cellulose activated by methods 1 - 3 are 367, 269 and 383 °C, respectively, indicating on the different structure and properties of cellulose activated by the different methods.

**Example 13. Effect of base type on trypsin activity and concentration of activating bound groups of cellulose prepared via methods 2 and 3.**

Cellulose activation via method 2 was performed accoding to example 12. Cellulose activation via method 3 was performed by transfering 1g dried cellulose to a flask containing 100 mg CDI (or 500 mg) in 10 ml of dried acetone. 2 ml of a desired base was then sometimes added dropwise for about 1-2 min. After 30 min reaction at room temperature the activated cellulose was washed extensively with dried acetone and then air-dried.

The dried activated cellulose prepared via methods 2 or 3 was added to 10 ml $NaHCO_3$ aqueous solution (0.1 M, pH - 8.5) containing 10 mg trypsin. The binding reaction continued for 18 h at room temperature. The trypsin conjugated cellulose was then washed extensively with $NaHCO_3$ aqueous solution (0.1 M, pH - 8.5), saline and water (fast) and then air-dried.

Table 5 demonstrates that the activated cellulose prepared via method 2 possess significantly increased concentration of bound activating groups compared to that prepared via method 3, i.e. 394 and 2.5 mg/g (cell), respectively. In spite of this the activity of trypsin conjugated cellulose prepared by method 2 is significantly lower than that prepared by method 3 ( 75 % lower when casein is the substrate). Table 5 also demonstrates that addition of a base during the activation of cellulose

via method 3 significantly decreased the amount of bound activating groups and the activity of the conjugated cellulose. Contrarily, tables 1 and 2 demonstrate that addition of a base such as TEA during the activation of cellulose via method 1 increased usually the activity of the conjugated cellulose.

**Example 14. Effect of CDI amount on the activity of trypsin conjugated to cellulose prepared via methods 2 and 3.**

Activation of cellulose was performed according to example12 - method 3, substituting activation time of 1 h for 30 min and under different CDI concentrations. Tables 6 and 7 show that maximal activity of conjugated trypsin was obtained for CDI concentration of 0.1 g/g cellulose. Increasing or decreasing of this concentration of CDI leads to a systematic decrease in trypsin activity.

**Example 15. Effect of trypsin concentration, CDI amount and activation time on the concentration of trypsin bound to cellulose prepared via methods 2 and 3.**

Activation of cellulose via method 2 was performed according to example 12 . Activation of cellulose via method 3 was performed according to example 14, by changing the CDI concentration or activation time. The dried activated cellulose prepared via methods 2 or 3 was added to 10 ml of $NaHCO_3$ aqueous solution (0.1 M, pH - 8.5) containing different trypsin concentration. The binding reaction continued for 18 h at room temperature. The trypsin conjugated cellulose was then washed extensively with $NaHCO_3$ aqueous solution (0.1 M, pH - 8.5), saline and water (fast) and then air-dried.

Table 8-A demonstrates that under similar trypsin concentration, i.e. 20 - 40 mg/g (cell) the amount of bound enzyme to cellulose prepared via methods 2 and 3 is similar, 3.7 - 3.8 mg/g (cell).

Table 8-B shows that increasing of CDI concentration of cellulose activated by method 3 resulting in increased amount of bound trypsin to cellulose, i.e. increasing the CDI concentration from 10 to 200 mg/g (cell) leads to increasing of bound trypsin concentration from 0.7 to 5.2 mg/g(cell).

Table 8-C shows that increasing the activation period of time above 15 min does not effect the amount of bound trypsin, i.e. for activation time of 5 min the concentration of bound trypsin is 0.5 mg/g (cell) and for activation time above 15 min up to 90 min the concentration of bound trypsin is similar, ca. 3.3 mg/g (cell).

**Example 16. Effect of lysozyme concentration on the amount of lysozyme bound to cellulose prepared via methods 2 and 3.**

Activation of cellulose via methods 2 and 3 was performed according to example 12. The binding of lysozyme to the activated cellulose was performed according to example 15 substituting trypsin for lysozyme. Table 9 demonstrates that under similar lysozyme concentration similar amount of lysozyme is bound to cellulose prepared via methods 2 or 3.

**Example 17. Effect of lysozyme concentration and pH on the activity of lysozyme bound to cellulose prepared via methods 2 and 3.**

Activation and binding of lysozyme to cellulose prepared via methods 2 and 3 were performed according to example 16. Figure 13 demonstrates that at pH-7.5 at each concentration of lysozyme the activity of bound lysozyme prepared via methods 2 and 3 is similar. Figure 13 also shows that
at pH-7.5 the activity of bound lysozyme prepared via methods 3 is higher than that prepared at pH-6.5.

**Example 18. Effect of metal salts on the activity of collagenase bound to cellulose via method 3.**

Activation of cellulose via methods 2 and 3 was performed according to example 12. The binding of collagenase to the activated cellulose was performed according to example 13 substituting 10 mg trypsin for 40 mg collagenase and adding different concentrations of metal salts to the bicarbonate buffer. Table 10 demonstrates that the bound collagenase prepared via method 3 is not active at all towards gelatin gel in absence or in presence of $CaCl_2$ in the reaction solvent

($NaHCO_3$ 0.1 M pH-8.5), e.g. after 22h the % of non-hydrolyzed gel remained 100%. On the other hand, the bound collagenase prepared by method 2 is active also in absence of metal salts, e.g after 22 h, the % of non-hydrolyzed gelatin gel was 35 % . Table 10 also shows that addition of metal salts such as $ZnCl_2$ or $CoCl_2$ significantly increased the activity of the bound collagenase prepared by method 3, i.e. in presence of 0.18 mmol $ZnCl_2$ or 0.13 mmol $CoCl_2$ the % gelatin gel remained after 22 h was 14% and 0% (all the gel hydrolyzed), respectively. Similar conclusions were obtained by using FALGPA as a substrate for the bound collagenase, as shown in table 11.

**Example 19.**

Example 1 - method 3 (CDI) was repeated substituting the 0.25 g cellulose dressing and 5 mg trypsin for 1 g cellulose and 10 mg trypsin. The protein bicarbonate solution (2.5 ml) was sqweezed on the cellulose dressing and then wrapped with aluminum foil and incubated for 24 h at room temperature. The cellulose was then removed from the aluminium and washed according to example 1. Similar results were obtained

**Example 20. Activity of trypsin and lysozyme conjugated to Sephadex via methods 2 and 3.**

Activation of dry Sephadex via methods 2 and 3 was performed according to example 12 substituting cellulose for Sephadex 200. The binding of trypsin or lysozyme to the activated Sephadex was performed according to example 15 in the presence of 10 mg trypsin or 20 mg lysozyme. The activity of trypsin bound to Sephadex prepared via method 3 towards BAEE and casein was ca. 3 to 6 times higher than that prepared via method 2. The activity of lysozyme bound to Sephadex prepared via methods 2 ond 3 towards Micrococcus lysodeikticus was similar.

**Example 21. Activation of polyhydroxy-polymers prepared via method 3 with CDT.**

Examples 10-14 repeated substituting CDI for CDT. Similar results were obtained.

**Example 22. Blocking residual bound imidazolyl groups with different reagents.**

Activation of cellulose via method 3 was performed according to example 12. The dried activated cellulose was added to 10 ml of $NaHCO_3$ aqueous solution (0.1 M, pH - 8.5) containing 10 mg trypsin. The binding reaction continued for ca. 10 h at room temperature. Then, residual bound imidazolyl groups were blocked by dissolving in the bicarbonate buffer various amino ligands, e.g. glycine, ethanol amine, polyethylen glycol containing terminal amine group, etc. The reaction continued then for another 12 h. The blocked trypsin conjugated cellulose was then washed extensively with $NaHCO_3$ aqueous solution (0.1 M, pH - 8.5), saline and water (fast) and then air-dried.

Studies in our laboratory demonstrate that the nature of the conjugated polymer was influenced by the blocking reagents, i.e. blocking the conjugated cellulose with glycine or polyethylene glycol containing terminal amine group increased its hydrophilicity as indicated by a water drop which placed on the surface of the different conjugated polymers. This kind of more hydrophilic bioactive dressings should enhanced the wound healing process.

**Example 23. Removal of specific antibodies from immunized serum by protein conjugated cellulose prepared via methods 1 and 3.**

Each 10 ml of goat serum containing goat anti human albumin antibodies was circulated (10 ml/min) at room temperature for 10 min through a column containing 1 g of human albumin conjugated cellulose dressings prepared via methods 1 or 3 according to example 12. The columns were then washed extensively with saline. The antibodies adsorbed to each column were then eluted with 0.2 M glycine HCl buffer at pH 2.4 and returned to neutral pH. The amount of antibodies adsorbed to the conjugated cellulose prepared via method 3 was approximately 3 times higher than that prepared via method 1.

**Example 24. Preparation of multi-enzyme conjugated cellulose dressings.**

Activation of cellulose via method 3 was performed according to example12. The protein binding procedure was similar to experiment 13 substituting 10 mg trypsin for 10 mg trypsin and 15 mg lysozyme.

Studies at our laboratory showed that 3 mg of trypsin and 3 mg of lysozyme were bound to each 1g cellulose and that the activity of each bound enzyme is between 80% to 90% of the activity of the enzymes which bound separately.

**Example 25. Stability of the protein conjugated cellulose.**

Each sample containing 50 mg of cellulose conjugated with proteins such as trypsin, lysozyme, collagenase and albumin was placed at room temperature in PBS (0.1 M pH - 7.4) for 48 h. The samples were then removed from the PBS solution.

Studies at our laboratory show that the activity of the conjugated protein did not change during the experiment period. Also, leakage of bound proteins into the PBS solution was not detected by using the method of Lowry et al (see J. Biol. Chem. 193, 265 (1951).

**Example 26. Sterilization of the bioactive polymers.**

Air dried cellulose dressings , containing each 100 mg cellulose conjugated with proteins such as trypsin and collagenase (prepared via method 3) hermetically packed in a nylon bag, have been sterilized by 2.5 Mrad gamma irradiation. The activity of the sterilized bioactive cellulose was between 80% to 100% of the non-irradiated bioactive dressings.

**Example 27. Preparation and activity of bioactive cellulose prepared via crosslinking reagents.**

Two steps method.

1 g of trypsin conjugated cellulose dressing prepared according to method 3 was soaked at room temperature for 3 hours in 2.5 ml of 0.066 M aqueous phosphate buffer, pH 6.25 containing 0.5% glutaraldehyde. Unbound GA was then washed extensively with the same buffer, saline and fast wash with distilled water. The formed glutaraldehyde modified trypsin-cellulose conjugate was then dryed at room temperature and then soaked for 6 hours in 2.5 ml of 0.1 M bicarbonate buffer, pH 8.5 containing 80 mg trypsin. Unbound trypsin was then removed by extensive washing with the same buffer. The bioactive dressing was then washed with saline and then fast wash with distilled water and then was air-dried.

One step method.

1 g of trypsin conjugated cellulose dressing prepared according to method 3 was soaked at room temperature for 5 min in 2.5 ml of 0.1 M aqueous bicarbonate buffer, pH 8.5 containing 4% trypsin (w/v). After 5 min, 2.5 ml of 0.066 M aqueous phosphate buffer, pH 6.25 containing 1% GA was added and the incubation was continued at room temperature for 18 h. Unbound trypsin was then removed by extensive washing with bicarbonate buffer, saline and then fast wash with distilled water and then air dried.

Studies at our laboratory showed that the amount and activity (per g cellulose) towards BAEE and casein of trypsin bound to these dressings prepared via one or two steps couling reactions were similar and approximately ten times higher than that prepared by method 3 in absence of a crosslinker reagent.

Similar results were obtained by performing similar experiment substituting trypsin for lysozyme.

**Example 28. In-vivo trials: treatment of wounds with collagenase-cellulose dressings prepared via methods 1 -3.**

Burn wounds formed on 15 guinea pigs, according to the description in materials and methods, were treated immediately after burning by bioactive dressings

composed of cellulose bound with collagenase prepared according to methods 2 and 3. Each bandage which consisted of 100 mg air-dried cellulose dressing was first soaked in saline solution (or in Tricine buffer). The wet bandages were then placed on the burn wounds and covered by nylon, thereby preventing evaporation of water. The bandages were replaced after 24 and 48 hours. The following studies were carried out during treatment of burn wounds: A. Visual control of the cleaning of the burn wounds, including photomicrographs; B. Measurement of wounds area cleaned from necrotic tissue, see table 12.

C. Activity of bound enzymes dressings toward gelating gel, before and after removal of the dressing from the wounds - in this case it was found that activity was substantially maintained in bandages prepared by methods 2 and 3 and that the superiority order is method 3 > Method 2.

These studies clearly demonstrated the superiority of the dressings prepared by method 3, as compared with the dressings prepared by the other methods.

Table 1: Activity of trypsin and lysozyme covalently bound to cellulose via different activating reagents.*

| Activating Reagent | Base | Relative Activity (% from CDI) | | |
|---|---|---|---|---|
| | | Lysozyme | Trypsin | |
| | | (towards micrococcus) | BAEE | Casein |
| CDI | - | 100 | 100 | 100 |
| Tosyl chloride | - | 10.3 | 10.5 | 5.9 |
| Tosyl chloride | TEA | 41 | 19.7 | 13.8 |
| | | | | |
| Tresyl chloride | - | 10.8 | 12.6 | 6.6 |
| Tresyl chloride | TEA | 42.6 | 21.1 | 19.7 |
| | | | | |
| NPC | - | 22.2 | 30 | 15.1 |
| NPC | TEA | 45.1 | 67 | 55.9 |
| | | | | |
| CNBr | TEA | 43.4 | 41.4 | 15.8 |

*Activity was calculated per 1 g cellulose

Table 2. Specific activity of trypsin and lysozyme covalently bound to cellulose via different activating reagnets.*

| Activating Reagent | Base | Relative Activity (% from CDI) | | |
|---|---|---|---|---|
| | | Lysozyme | Trypsin | |
| | | (towards micrococcus) | BAEE | Casein |
| CDI | - | 100 | 100 | 100 |
| Tosyl chloride | - | 21.8 | 11 | 6.1 |
| Tosyl chloride | TEA | 54.6 | 12.7 | 9 |
| | | | | |
| Tresyl chloride | - | 19.1 | - | 12.8 |
| Tresyl chloride | TEA | 34.1 | 16.3 | 15.4 |
| | | | | |
| NPC | - | 15.9 | 30 | 39.2 |
| NPC | TEA | 25.8 | 55.6 | 46.1 |
| | | | | |
| CNBr | TEA | 26 | 45.7 | 17.7 |

*Activity was calculated per mg bound enzyme

Table 3. Swelling level of cellulose in various environments.

| Environment | ΔV(%) |
|---|---|
| Acetone (2 ml) | 0 |
| Pyridine (2 ml) | 44 |
| $H_2O$ (2 ml) | 13 |
| Pyridine (1 ml) + acetone (1 ml) | 12 |
| CDI (50 mg) in acetone (2 ml) | 4 |
| CDI (100 mg) in acetone (2 ml) | 11 |
| CDI (200 mg) in acetone (2 ml) | 15 |
| Imidazole (20 mg) in acetone (2 ml) | 15 |
| Imidazole (75 mg) in acetone (2 ml) | 23 |

Table 4. TGA behavior of cellulose and activated cellulose prepared by methods 1-3.

| Dressing type | Dpeak (°C) (point of bend) |
|---|---|
| Cellulose | 373 |
| Activated cellulose prepared by method 1 | 367 |
| Activated cellulose prepared by method 2 | 269 |
| Activated cellulose prepared by method 3 | 383 |

Table 5. Effect of base type on trypsin activity and concentration of bound activating groups of cellulose prepared by methods 2 and 3.*

| Activating Reagent | Base | Concentration of bound activating groups mg/g(cell) | Relative Activity (% from CDI) | |
|---|---|---|---|---|
| | | | BAEE | Casein |
| Tosyl chloride (method 2) | Pyridine (for pretreatment) | 394 | 54 | 25 |
| CDI | - | 2.5 | 100 | 100 |
| CDI (500 mg) | - | 2.2 | - | - |
| CDI | TEA | 0.3 | 67 | 28 |
| CDI | Triethanol amine | 0.4 | 3 | 12 |
| CDI | Pyridine | 0.8 | 95 | 57 |

*Activity was calculated per 1 g cellulose.

Table 6. Effect of CDI amount on the activity towards BAEE and casein of trypsin conjugated to cellulose prepared via methods 2 and 3.*

| [CDI] (mg) | Relative Activity (% from 100 mg CDI) | |
|---|---|---|
| | BAEE | Casein |
| 0 | 0 | 0 |
| 10 | 78 | 39 |
| 20 | 86 | 61 |
| 50 | 96 | 76 |
| 100 | 100 | 100 |
| 200 | 100 | 95 |
| 500 | 89 | - |
| 1000 | 84 | 21 |

* Activity was calculated per 1 g cellulose

Table 7. Effect of CDI amount on the activity towards gelatin gel of trypsin conjugated to cellulose prepared via methods 2 and 3.

| Activation method | [CDI] (mg) | Activity (% of non-hydrolyzed gel after 22h) |
|---|---|---|
| 2 | - | 72 |
| 3 | 0 | 100 |
| 3 | 10 | 58 |
| 3 | 20 | 39 |
| 3 | 50 | 38 |
| 3 | 100 | 24 |
| 3 | 200 | 38 |
| 3 | 500 | 45 |
| 3 | 1000 | 63 |

Table 8. Effect of trypsin concentration, CDI amount and activation time on the concentration of trypsin bound to cellulose via methods 2 and 3.

| | A | | B | | C | |
|---|---|---|---|---|---|---|
| Method | [Trypsin] mg/g(cell) | [Bound Trypsin] mg/g(cell) | [CDI] mg | [Bound Trypsin] mg/g(cell) | Activation Time min | [Bound Trypsin] mg/g(cell) |
| 2 (Tosyl chloride) | 20-40 | 3.8 | - | - | - | - |
| 3 | 6 | 2.1 | 10 | 0.7 | 5 | 0.5 |
| 3 | 10 | 3.0 | 20 | 2.9 | 15 | 3.3 |
| 3 | 20 | 3.8 | 50 | 3.3 | 30 | 3.1 |
| 3 | 40 | 3.7 | 100 | 4.1 | 60 | 3.3 |
| 3 | - | - | 200 | 5.2 | 90 | 3.7 |

Table 9. Effect of lysozome concentration on the amount of lysozome bound to cellulose prepared via methods 2 and 3.

| [Lysozyme] mg/g(cell) | [Bound Lysozyme] mg/g(cell) | |
|---|---|---|
| | Method 2 | Method 3 |
| 10 | 3.0 | 2.5 |
| 30 | 3.0 | 3.3 |
| 60 | 4.6 | 4.0 |
| 100 | 4.3 | 3.7 |

Table 10. Effect of metal salts on the activity toward gelatin gel of collagenase bound to cellulose via methods 2 and 3.

| Activation | $[M^{2+}]$ (mmol) | $\frac{[M^{2+}]}{[\text{Bound imidazolyl groups}]}$ | Activity (% of non-hydrolyzed gel) after 22 h) |
|---|---|---|---|
| 2 | - | - | 35 |
| 3 | - | - | 100 |
| 3 | $CaCl_2$ 0.18 | 7 | 100 |
| 3 | $CaCl_2$ 3.6 | 138 | 100 |
| 3 | $ZnCl_2$ 0.09 | 3.5 | 67 |
| 3 | $ZnCl_2$ 0.18 | 7 | 14 |
| 3 | $ZnCl_2$ 0.36 | 14 | 16 |
| 3 | $(CaCl_2 + ZnCl_2)$ $(0.18 + 0.18)$ | 7 | 61 |
| 3 | $CoCl_2$ 0.13 | 5 | 0 |
| 3 | $CoCl_2$ 0.26 | 10 | 0 |

Table 11. Effect of metal salts on the activity towards FALGPA of collagenase bound to cellulose via method 3.*

| Activation method | $[M^{2+}]$ (mmol) | %)from $CoCl_2$ sample) FALGPA |
|---|---|---|
| 2 | - | 50 |
| 3 | $ZnCl_2$ - 0.36 | 75 |
| 3 | $CoCl_2$ - 0.13 | 106 |

*Activity was calculated per 1 g cellulose

Table 12. Dynamics of cleaning of burn wounds from necrotic tissue, expressed as % of total wound area* cleaned from necrotic tissue.

| Time of Treatment (h) | Method of Preparation | | |
|---|---|---|---|
| | Method 1 | Method 2 | Method 3 |
| 10 | 0 | 3 -5 | 15 - 20 |
| 24 | | | |
| 48 | | | |

* Values are average of experiments carried out with 12 guinea pigs, 3 in each method.

**Claims:**

1. A method to prepare a bioactive polymer by covalently binding at least one amino group containing ligand to at least one polymer containing a plurality of free hydroxyl groups, said method comprising the following steps:

(I) reacting the at least one polymer with an appropriate activating agent;

(ii) reacting the resultant activated polymer in aqueous solution with desired amino group containing ligands;

(iii) blocking by reaction or removing by hydrolysis residual polymer bound-ligand unreacted, activating groups;

and wherein the said activating agent and/or leaving by-products formed by step (i) and/or by step (ii) and/or by step (iii), are swelling reagents of the polyhydroxy polymer.

2. A method according to claim 1 wherein the polyhydroxy polymer is cellulose.

3. A method according to claim 1 wherein the polyhydroxy polymer is a derivative of cellulose.

4. A method according to claim 1 wherein the polyhydroxy polymer is polysaccharide other than cellulose such as dextrane (Sephadex).

5. A method according to claim 1 wherein the polyhydroxy polymer is polyvinylalcohol.

6. A method according to claim 1 wherein the activating reagent is a carbonylating reagent.

7. A method according to claim 1 wherein the activating reagent is 1,1-carbonyldiimidazole

8. A method according to claim 1 wherein the activating reagent is 1,1-carbonyldi-1,2,4-triazole.

9. A method according to claim 1 wherein the amino ligand is a protein.

10. A method according to claim 1 wherein the amino ligand is trypsin.

11. A method according toclaim 1 wherein the amino ligand is lysozyme.

12. A method according to claim 1 wherein the amino ligand is collagenase.

13. A method according to claim 1 wherein the amino ligand is albumin.

14. A method according to claim 1 wherein the amino ligand is a protein and the blocking reagent is a compound containing at least one amine group such as glycine, ethanolamine, polyethyleneglycol containing a terminal amine, etc.

15. A method according to claim 1 wherein the polyhydroxy polymer is a powder, bead, bandage or like cover

16. A method according to claim 1 for wound healing applications.

17. A method according to claim 1 for removal of undesired compound from liquids.

18. A method according to claims 1 -15 including the additional step of reacting the said amino group containing ligand covalently bound to hydroxy containing polymer in the presence of non-conjugated protein in at least one step with a crosslinking reagent.

19. A method according to claim 18 wherein the crosslinking reagent is glutaraldehyde.

20. A powder, bead, bandage or like cover for application to wounds which has been manufactured in accordance with claims 1-15.

21. A cover for application to wounds according to claim 20 wherein the polyhydroxy polymer is cellulose.

22. A cover for application to wounds according to claim 20 wherein the polyhydroxy polymer is a derivative of cellulose.

23. A cover for application to wounds according to claim 20 wherein the polyhydroxy polymer is a polysaccharide other than cellulose such as dextrane (Sephadex).

24. A cover for application to wounds according to claim 20 wherein the polyhydroxy polymer is polyvinylalcohol.

25. A cover for application to wounds according to claim 20 wherein the activating reagent is a carbonylating reagent.

26. A cover for application to wounds according to claim 25 wherein the carbonylating reagent is 1,1-carbonyldiimidazole or 1,1-carbonyldi-1,2,4-triazole.

27. A cover for application to wounds according to claim 20 wherein the amino ligand is a protein.

28. A cover for application to wounds according to claim 27 wherein the protein is selected from trypsin, lysozyme, collagenase or albumin.

29. A cover for application to wounds according to claim 20 wherein the amino ligand is a protein and the blocking reagent is a compound containing at least one

amine group such as glycine, ethanolamine, polyethylene glycol containing a terminal amine, etc.

30. A powder, bead, bandage or like cover according to claims 20 - 29 for removal of undesired compound from liquids.

31. A powder, bead, bandage or like cover for application to wounds which has been manufactured in accordance with claim 18.

32. A powder, bead, bandage or like cover for application to wounds which has been manufactured in accordance with claim 19.

33. A powder, bead, bandage or like cover according to claims 20-29 that have been further reacted in the presence of non-conjugated protein in at least one step with a crosslinking reagent.

34. A cover according to example 31 wherein the crosslinking reagent is glutaraldehyde.

FIG. 1

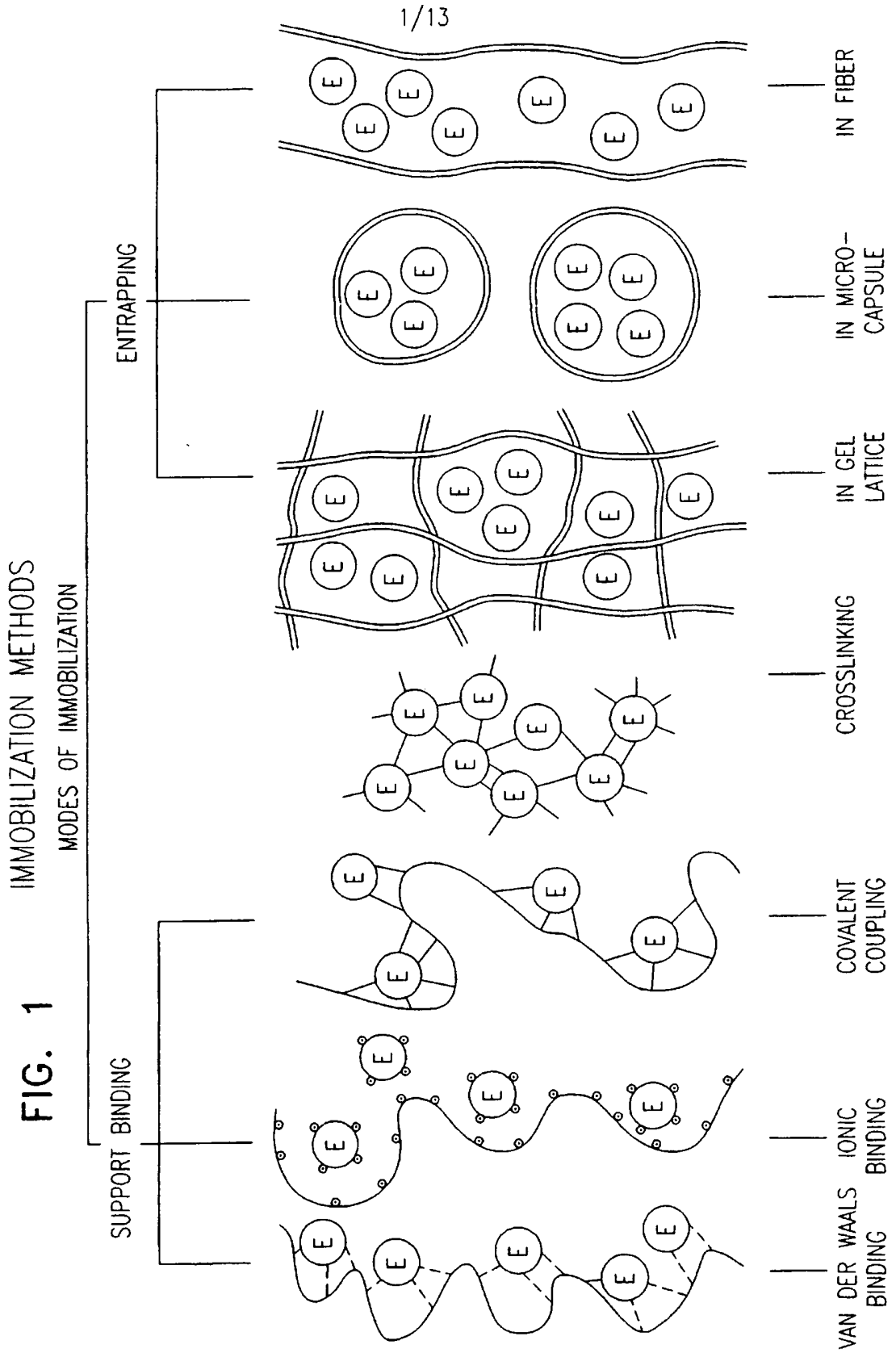

IMMOBILIZATION METHODS
MODES OF IMMOBILIZATION
ENTRAPPING
SUPPORT BINDING
IN FIBER
IN MICRO-CAPSULE
IN GEL LATTICE
CROSSLINKING
COVALENT COUPLING
IONIC BINDING
VAN DER WAALS BINDING

FIG. 2

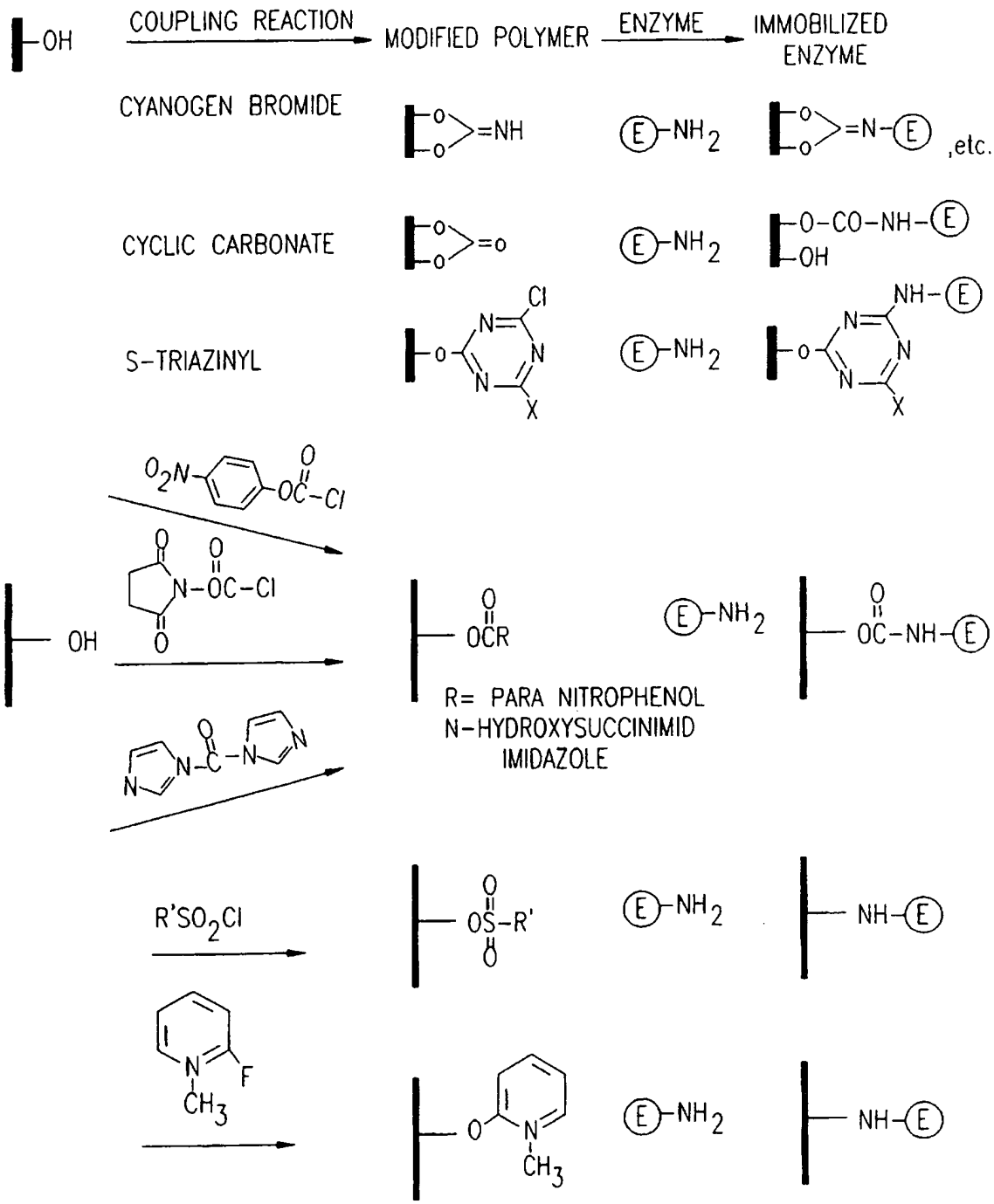

OH
COUPLING REACTION
MODIFIED POLYMER
ENZYME
IMMOBILIZED ENZYME
CYANOGEN BROMIDE
=NH
E-NH2
=N-E
,etc.
CYCLIC CARBONATE
=O
O-CO-NH-E
OH
S-TRIAZINYL
Cl
X
NH-E
OCR
OC-NH-E
R= PARA NITROPHENOL
N-HYDROXYSUCCINIMID
IMIDAZOLE
R'SO2Cl
OS-R'
NH-E
CH3
F

FIG. 3

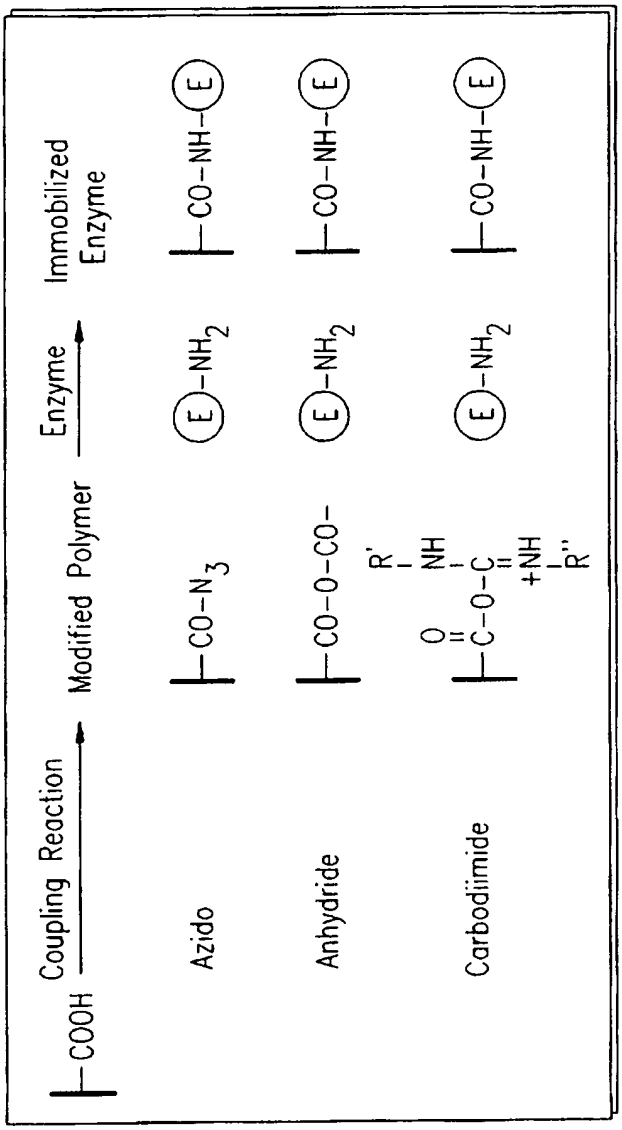
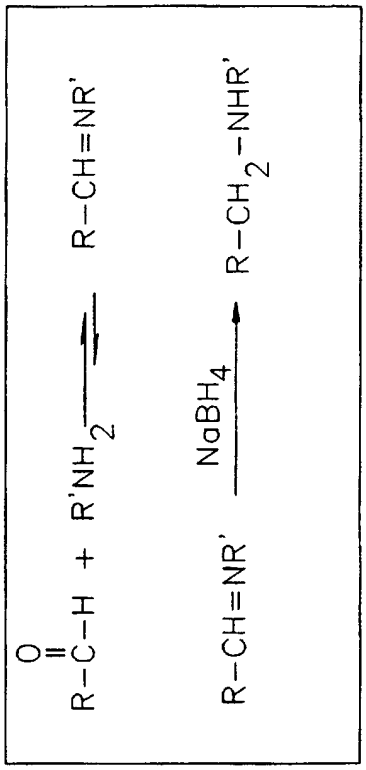

COOH
Coupling Reaction
Modified Polymer
Enzyme
Immobilized Enzyme
Azido
CO-N3
E-NH2
CO-NH-E
Anhydride
CO-O-CO-
R'
NH
Carbodiimide
O
C-O-C
+NH
R''
E-NH2
CO-NH-E

FIG. 4



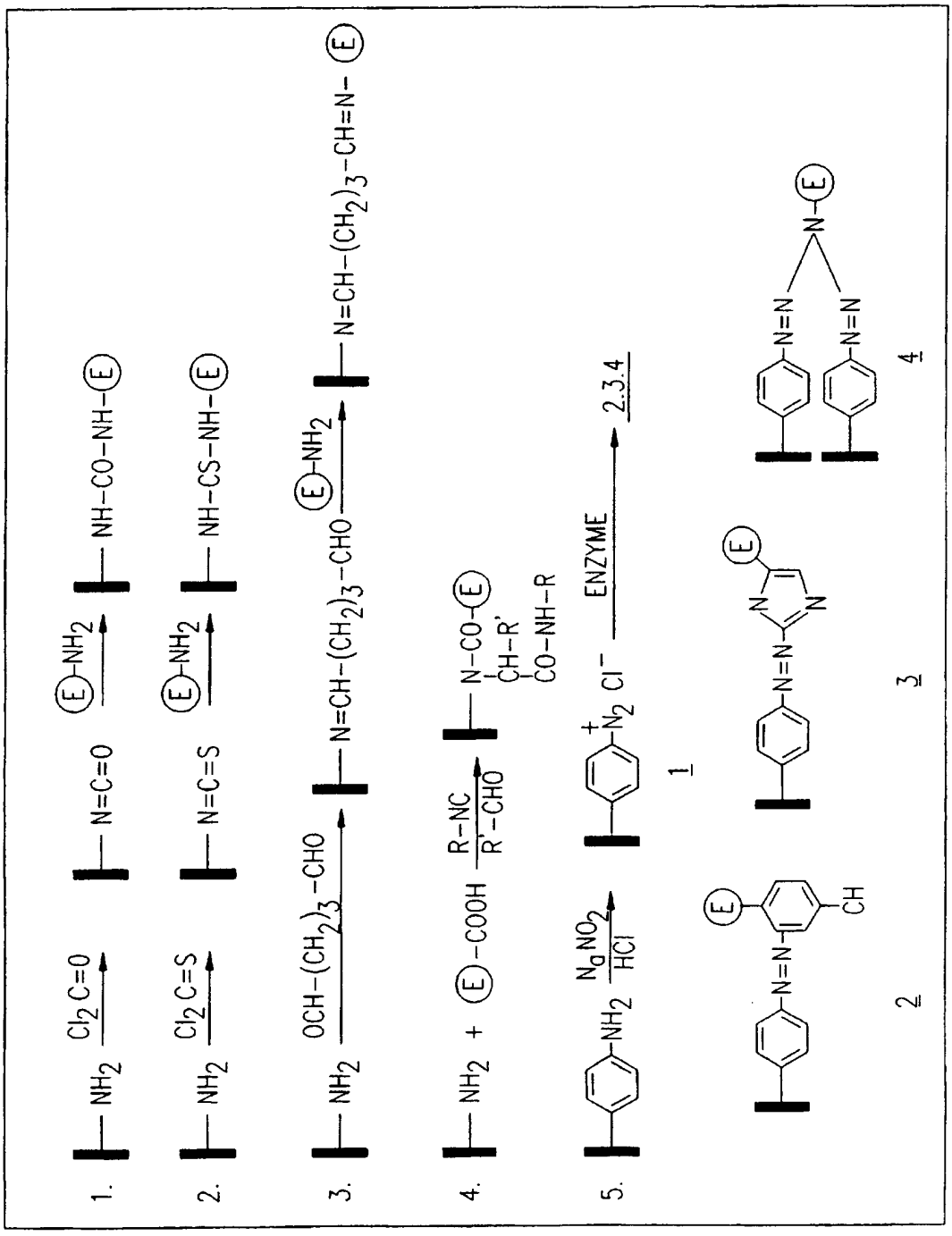

1. NH2 Cl2 C=O N=C=O E-NH2 NH-CO-NH-E
2. NH2 Cl2 C=S N=C=S E-NH2 NH-CS-NH-E
3. NH2 OCH-(CH2)3-CHO N=CH-(CH2)3-CHO E-NH2 N=CH-(CH2)3-CH=N-E
4. NH2 + E-COOH R-NC R'-CHO N-CO-E CH-R' CO-NH-R
5. NH2 NaNO2 HCl N2+ Cl- ENZYME 2.3.4
1
2
3
4

FIG. 5

FIG. 6

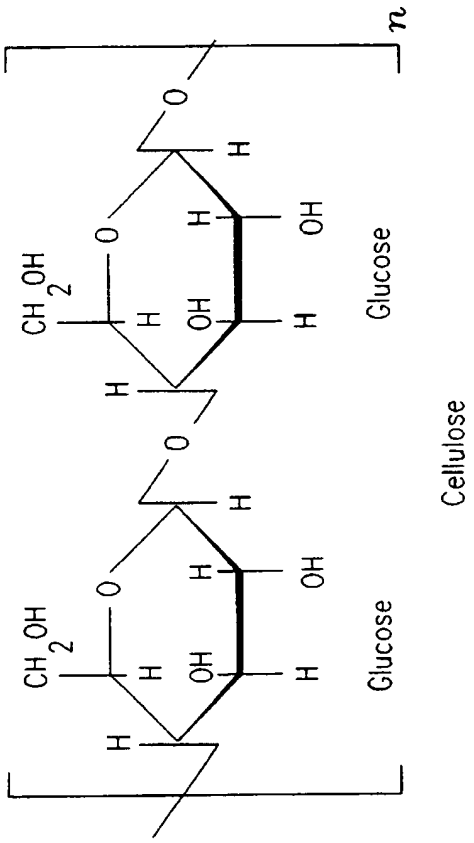

n
O
H
H
OH
O
CH2OH
H
OH
H
H
Glucose
O
H
H
OH
O
CH2OH
H
OH
H
H
Glucose
Cellulose

FIG. 7

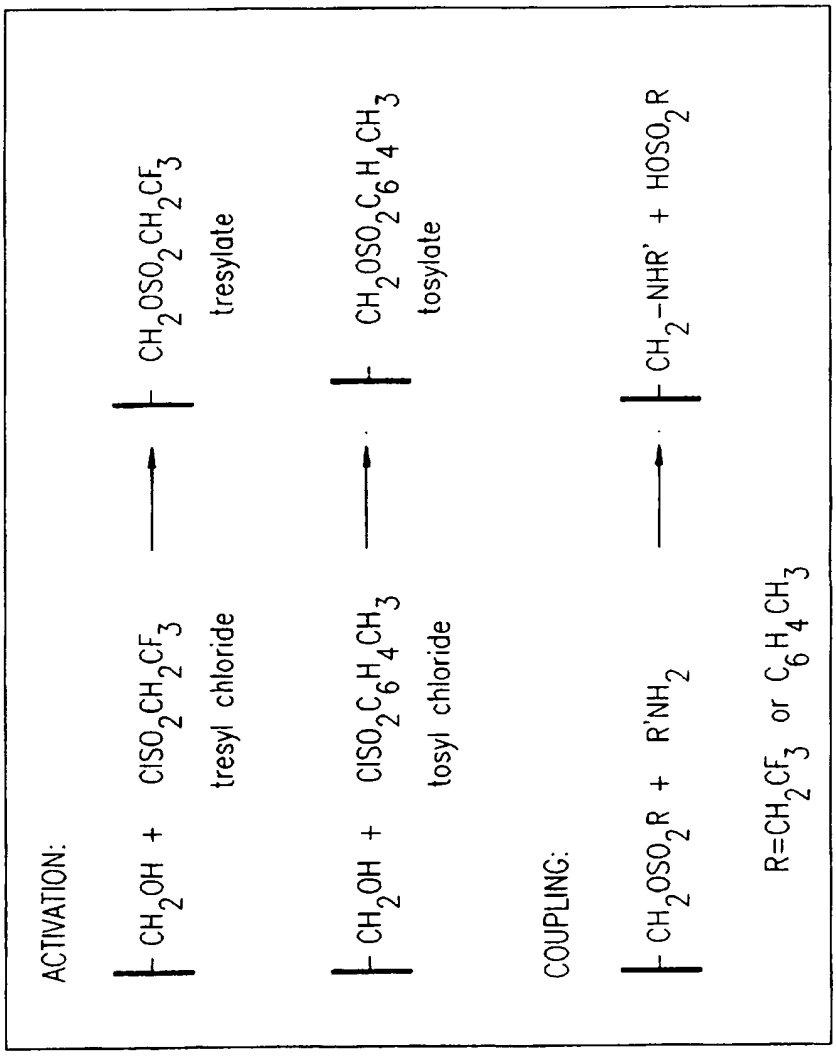
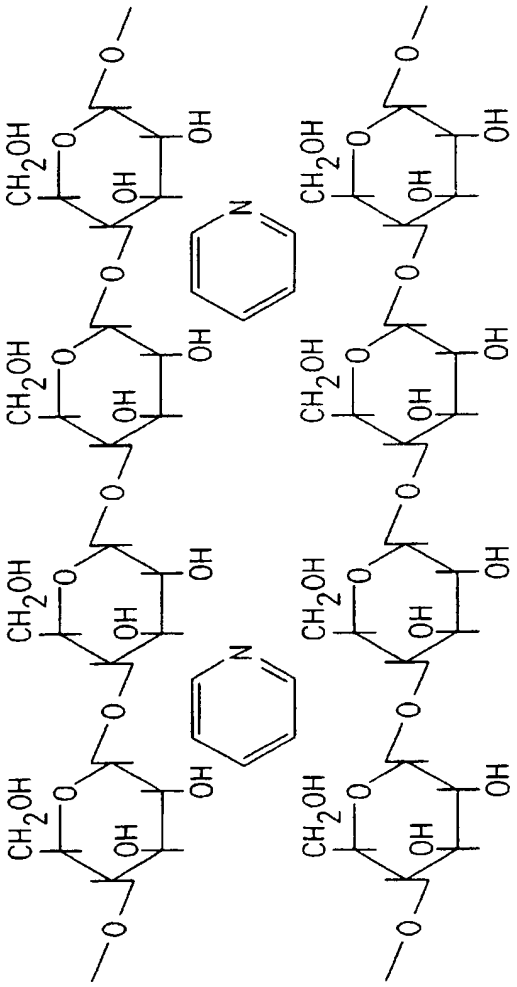

ACTIVATION:
$CH_2OH$ + $ClSO_2CH_2CF_3$ → $CH_2OSO_2CH_2CF_3$
tresyl chloride
tresylate
$CH_2OH$ + $ClSO_2C_6H_4CH_3$ → $CH_2OSO_2C_6H_4CH_3$
tosyl chloride
tosylate
COUPLING:
$CH_2OSO_2R$ + $R'NH_2$ → $CH_2-NHR'$ + $HOSO_2R$
R=$CH_2CF_3$ or $C_6H_4CH_3$

FIG. 8



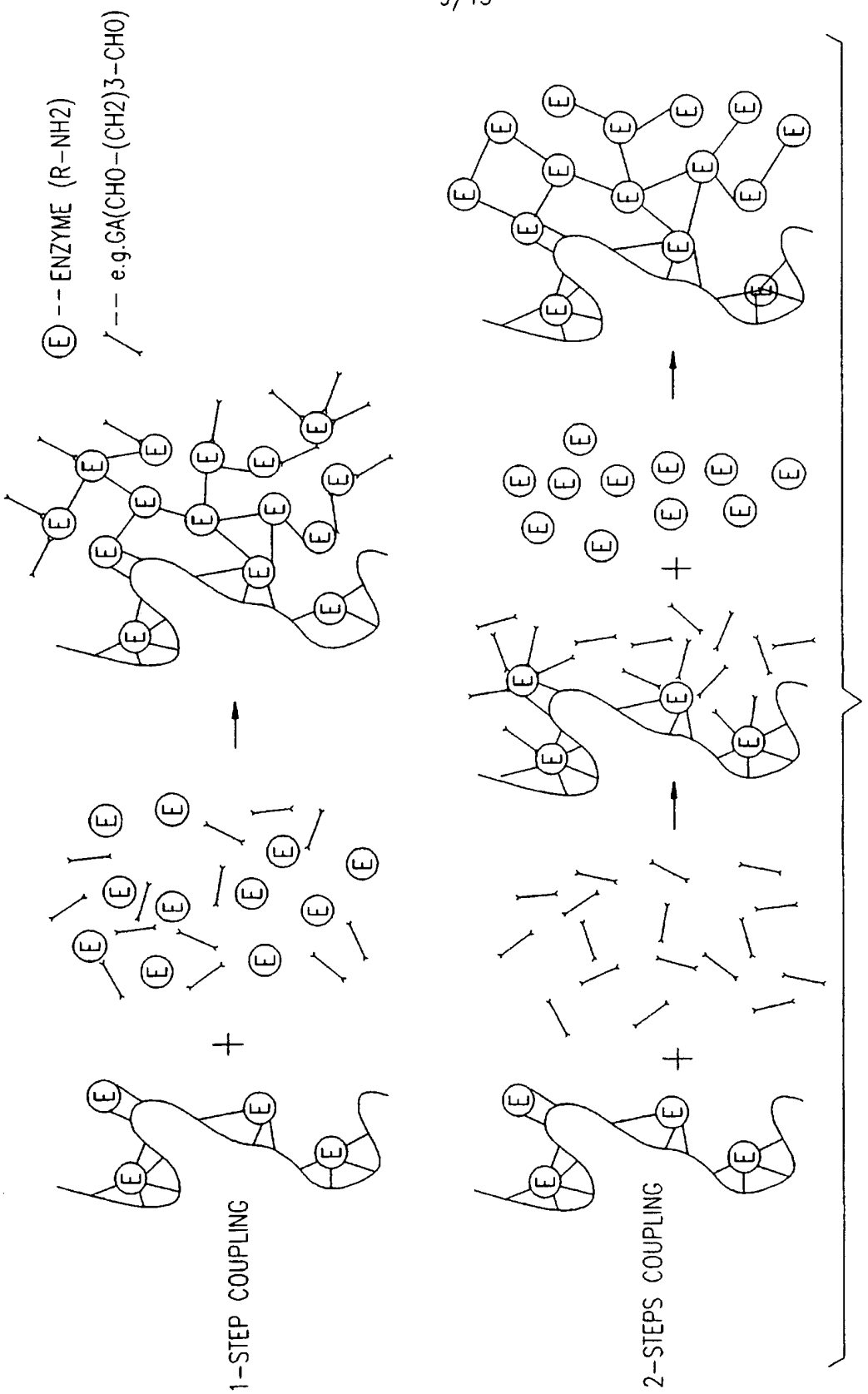

E -- ENZYME (R-NH2)
-- e.g.GA(CHO-(CH2)3-CHO)
1-STEP COUPLING
2-STEPS COUPLING

FIG. 9

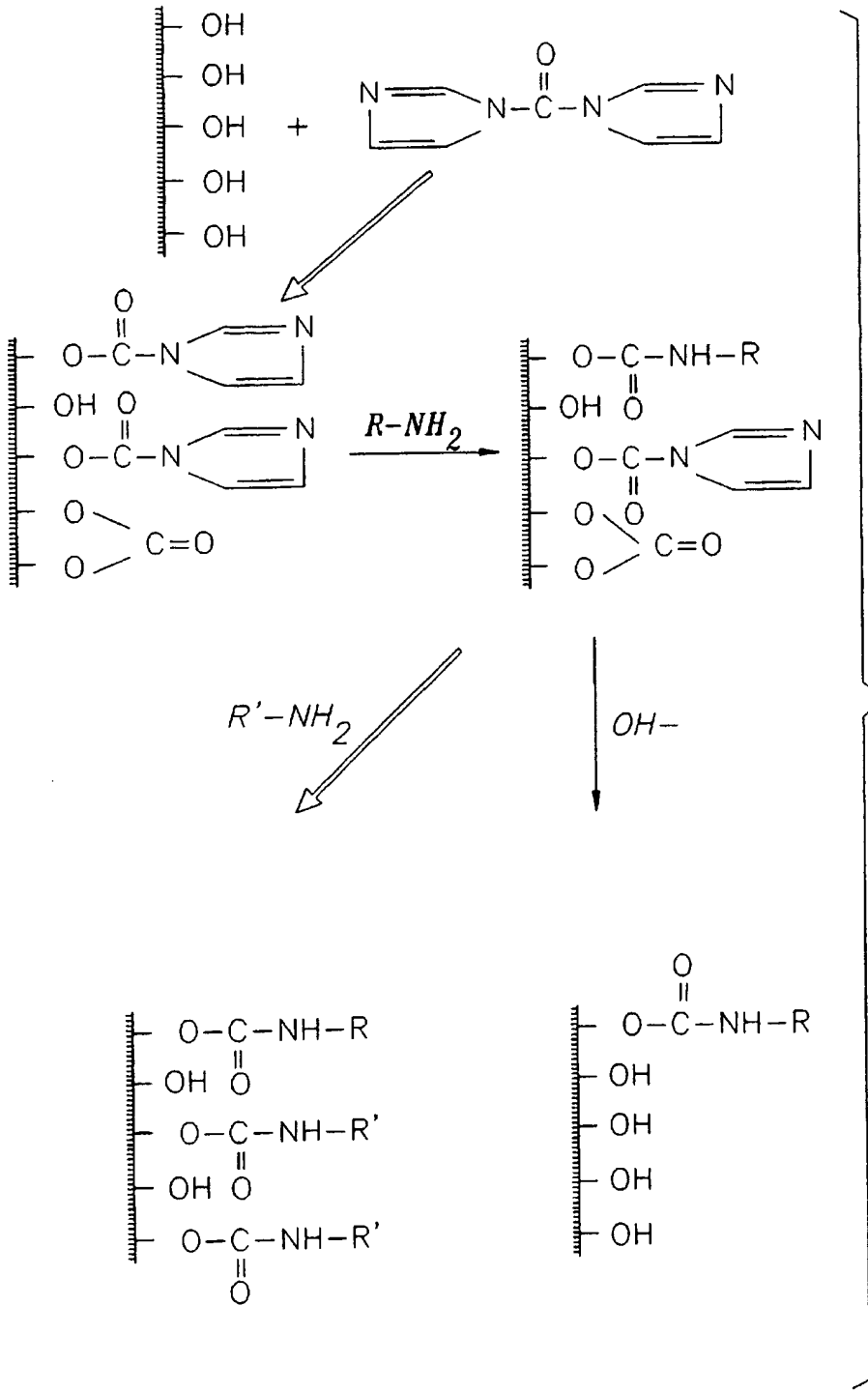



FIG. 10

FIG. 11

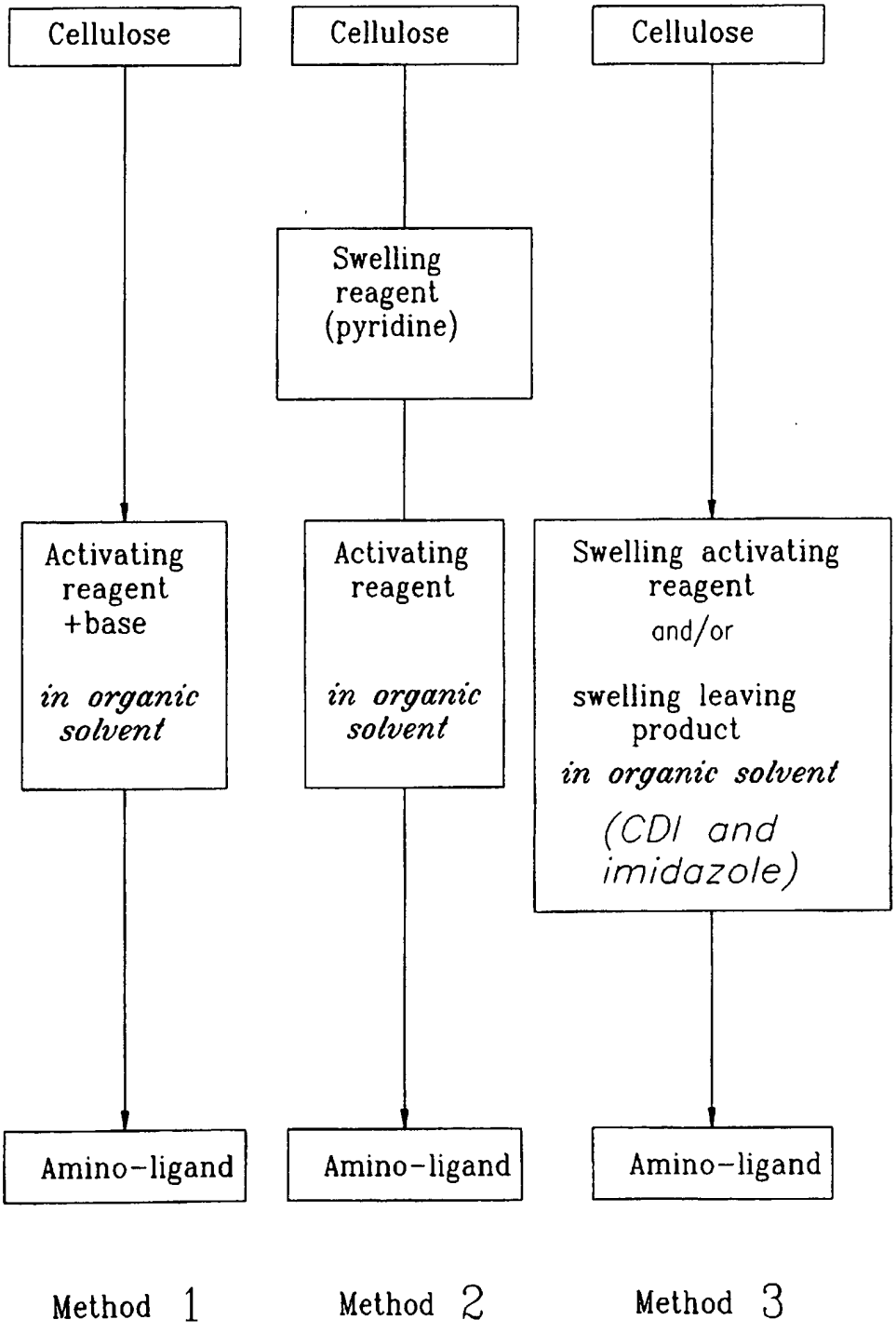

Cellulose
Cellulose
Cellulose
Swelling reagent (pyridine)
Activating reagent +base
in organic solvent
Activating reagent
in organic solvent
Swelling activating reagent
and/or
swelling leaving product
in organic solvent
(CDI and imidazole)
Amino-ligand
Amino-ligand
Amino-ligand
Method 1
Method 2
Method 3

FIG. 12

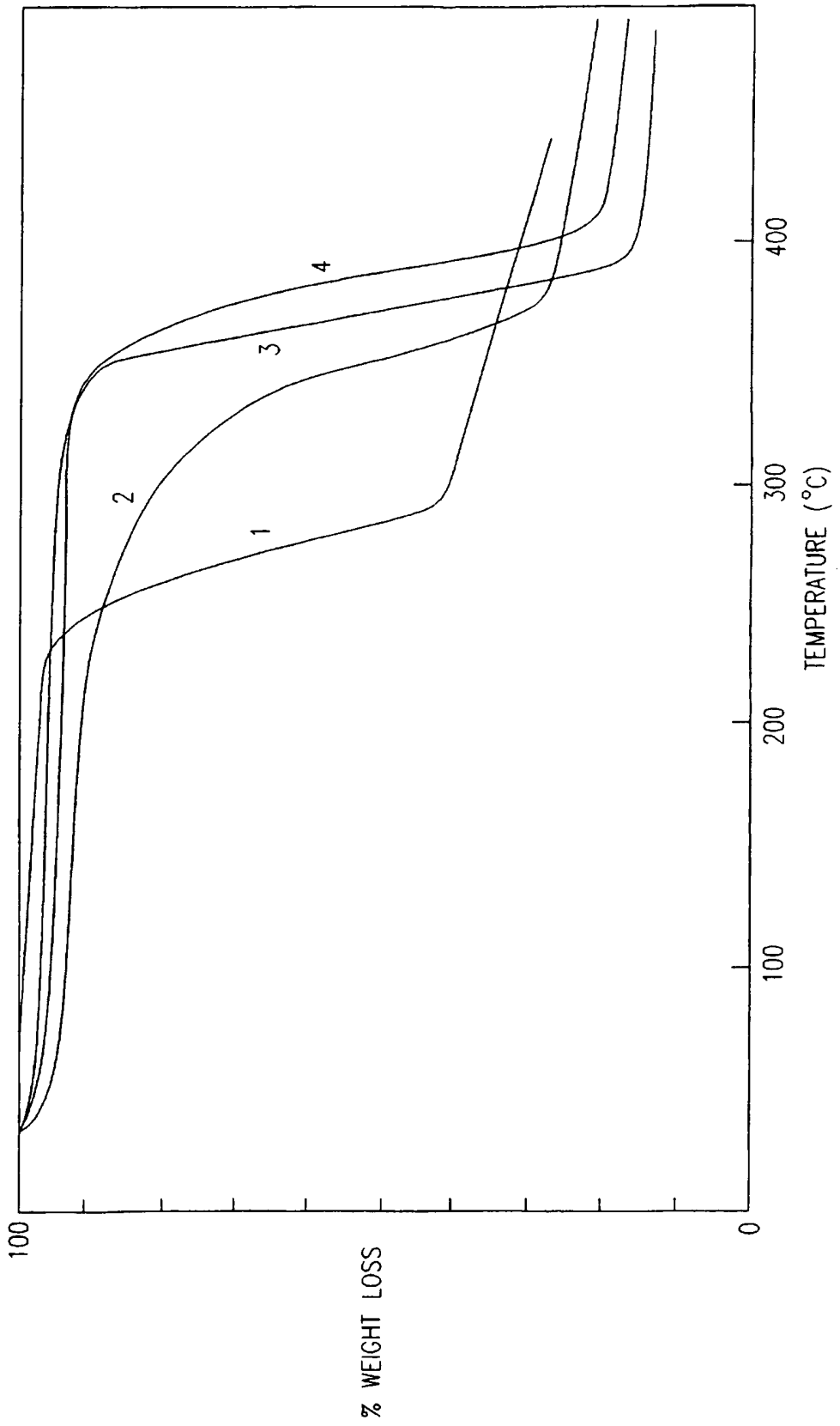
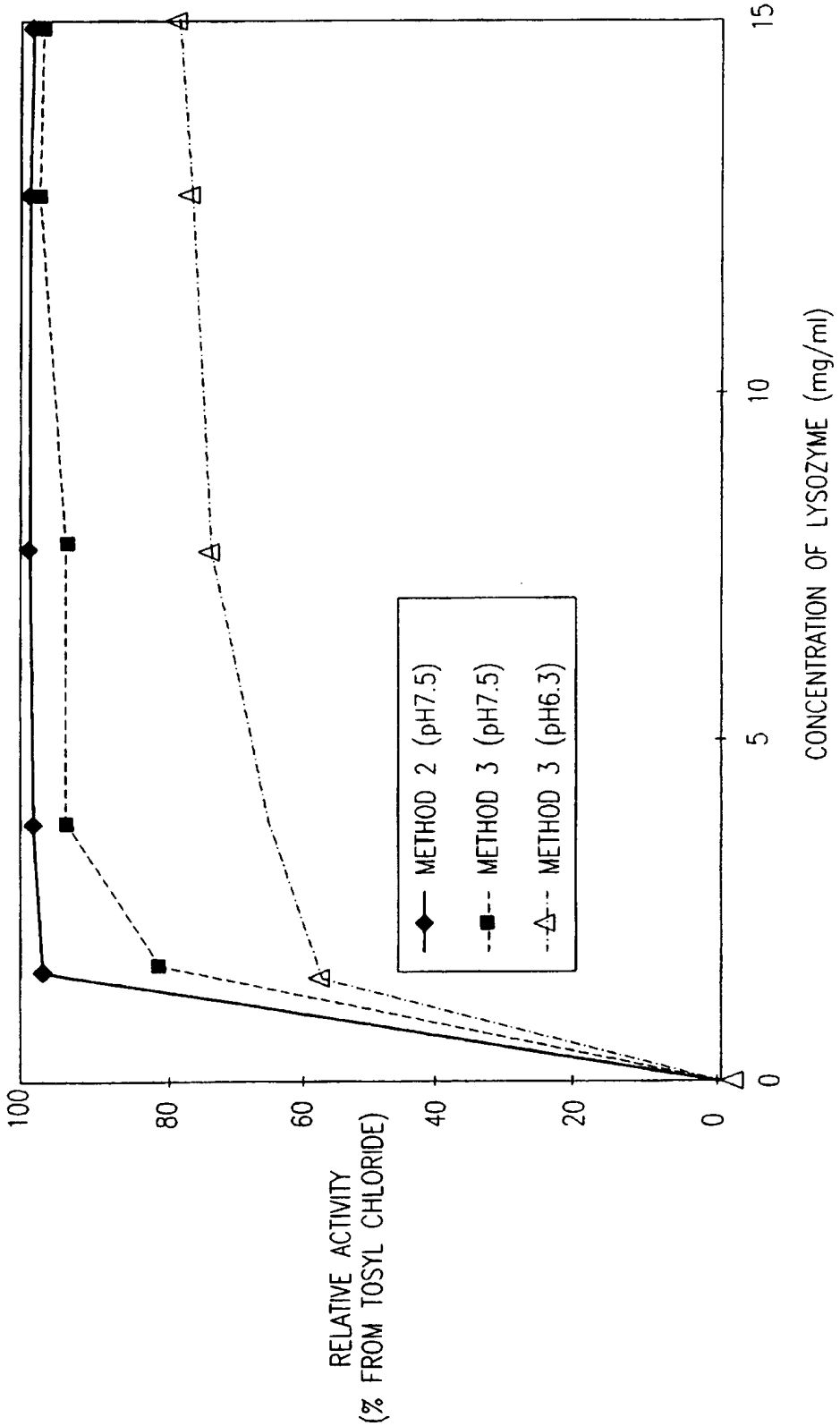


FIG. 13


RELATIVE ACTIVITY
(% FROM TOSYL CHLORIDE)
100
80
60
40
20
0
0
5
10
15
CONCENTRATION OF LYSOZYME (mg/ml)
METHOD 2 (pH7.5)
METHOD 3 (pH7.5)
METHOD 3 (pH6.3)